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NEW METHODS FOR CALCULATING METABOLIC RATE WITH SPECIAL REFERENCE TO PROTEIN METABOLISM

By J. B. de V. WEIR

From the Institute of Physiology, University of Glasgow

(*Received* 15 *May* 1948)

A curious fact in the estimation of metabolic rate by indirect calorimetry is that the normal 'exact' method of calculation is so cumbersome that the effect of protein metabolism is commonly ignored. Moreover, the total respiratory quotient is used to assign to the oxygen consumed a calorie value which is appropriate only to the non-protein respiratory quotient. This paper describes several ways in which the calculation can be simplified and shows how the effect of protein metabolism can be included with a minimum of trouble. The derivation of the calorie value of 1 litre of oxygen is the first step.

The calorie value of 1 *l. of oxygen*

If K kg.cal. is the calorie value of 1 l. of oxygen and R is the total R.Q., then, using the symbols and numerical values of Table 1, for 1 l. of oxygen consumed we have

$$\text{l. O}_2\text{ consumed} = x + y + z = 1, \tag{1}$$

$$\text{l. CO}_2\text{ produced} = R = x + 0{\cdot}802y + 0{\cdot}718z, \tag{2}$$

and

$$\text{kg.cal. liberated} = K = 5{\cdot}047x + 4{\cdot}463y + 4{\cdot}735z. \tag{3}$$

TABLE 1. Symbols and numerical values used in discussing the metabolism of carbohydrate, protein and fat

	Carbohydrate	Protein	Fat
R.Q.	1	0·802*	0·718†
Kg.cal. per litre of O_2	5·047‡	4·463*	4·735†
Vol. of O_2 metabolizing	x	y	z

* Data of Loewy modified by Lusk (1928).

† Cathcart & Cuthbertson (1931).

‡ Zuntz (1897).

Solving for K by first substituting for x from (1) in (2) and (3) we have

$$R = 1 - 0{\cdot}198y - 0{\cdot}282z, \tag{4}$$

and

$$K = 5{\cdot}047 - 0{\cdot}584y - 0{\cdot}312z, \tag{5}$$

and then substituting for z from (4) in (5) we have

$$K = 3{\cdot}941 + 1{\cdot}106R - 0{\cdot}365y. \qquad (6)$$

Since R is the total R.Q. and y is the number of litres of oxygen metabolizing protein per litre total oxygen consumption, the term $0{\cdot}365y$ represents the error in the usual method of calculation, and can be regarded as the correction for protein metabolism. When y is zero, R becomes the non-protein R.Q. and equation (6) then gives the calorie value of 1 l. of oxygen metabolizing carbohydrate and fat, and corresponds to the values in Cathcart & Cuthbertson's table (1931).

The protein correction can be made in several ways. Since 1 g. urinary nitrogen is equivalent to 5·941 l. of oxygen (Lusk, 1928) the correction is $0{\cdot}365 \times 5{\cdot}941 = 2{\cdot}17$ kg.cal./g. urinary nitrogen.

Alternatively, if the fraction of the total calorie production due to protein is p, the number of kg.cal. produced by protein metabolism per litre total oxygen consumption is pK, the volume of oxygen consumed is $pK/4{\cdot}463$ l. and so equation (6) can be rewritten as

$$K = 3{\cdot}941 + 1{\cdot}106R - 0{\cdot}365pK/4{\cdot}463,$$

which, on collecting terms in K, gives

$$K = (3{\cdot}941 + 1{\cdot}106R)/(1 + 0{\cdot}082p), \qquad (7)$$

or very nearly

$$K = (3{\cdot}941 + 1{\cdot}106R)\,(1 - 0{\cdot}082p). \qquad (8)$$

The protein correction is equal to a deduction of 1% when $p = 0{\cdot}123$, i.e. when 12·3% of the total calories arise from protein metabolism, and therefore the error in neglecting the effect of protein metabolism is 1% for each 12·3% of the total calories which arise from protein. Appropriate values of p can be used to suit special conditions; but in human metabolism p is usually about 1/8, and then equation (7) reduces very nearly to

$$K = 3{\cdot}9 + 1{\cdot}1R. \qquad (9)$$

In fact if the percentage of protein calories lies between 10 and 14 the maximum error in using equation (9) is less than 1 in 500.

The equation for the total heat output in a given time is

$$\text{total kg.cal.} = 3{\cdot}941 \times \text{l. } O_2 \text{ used} + 1{\cdot}106 \times \text{l. } CO_2 \text{ produced}$$
$$- \text{protein correction (e.g. } 2{\cdot}17 \times \text{g. urinary nitrogen)}, \qquad (10)$$

or

$$\text{total kg.cal.} = (3{\cdot}941 \times \text{l. } O_2 \text{ used} + \text{l. } CO_2 \text{ produced})/(1 + 0{\cdot}082p), \qquad (11)$$

or corresponding to equation (9)

$$\text{total kg.cal.} = 3{\cdot}9 \times \text{l. } O_2 \text{ used} + 1{\cdot}1 \text{ l. } CO_2 \text{ produced}. \qquad (12)$$

The carbon dioxide production and, in some cases, the oxygen consumption were determined by weight in many of the classical experiments. Indeed such is still the practice in some modern work.

The above formulae are easily modified to suit such data. For example, equation (12) becomes

$$\text{total kg.cal.} = 2{\cdot}729 \times \text{g. } O_2 \text{ used} + 0{\cdot}560 \times \text{g. } CO_2 \text{ produced.} \qquad (13)$$

The calorie value of 1 *l. of expired air*

In ordinary breathing, and also with all open types of respiratory apparatus used for determining energy expenditure, e.g. the Douglas bag, the calorie output is most simply found by multiplying the volume of expired air by the calorie value per litre of expired air.

Corresponding to each litre of expired air let O' l. be the volume of oxygen consumed, C' l. the volume of carbon dioxide produced and K' kg.cal. the heat liberated. Then, using the additional symbols shown in Table 2, we have

$$O' = (N_e O_i - O_e N_i)/100N_i, \qquad (14)$$

$$C' = (C_e N_i - N_e C_i)/100N_i, \qquad (15)$$

TABLE 2. Symbols and numerical values used in the description of indirect calorimetry

	CO_2	O_2	N_2
Percentage in inspired air	C_i (0·03)	O_i (20·93)	N_i (79·04)
Percentage in expired air	C_e	O_e	N_e

and, from (10), $K' = 3{\cdot}941O' + 1{\cdot}106C'$ -protein correction. Substituting for O' and C' and eliminating N_e by putting $N_e = 100 - C_e - O_e$ we get

$$K' = 1{\cdot}0432 - 0{\cdot}04984O_e + 0{\cdot}00063C_e - \text{protein correction,}$$

or

$$K' = (1{\cdot}0432 - 0{\cdot}04984O_e + 0{\cdot}00063C_e)/(1 + 0{\cdot}082p). \qquad (16)$$

These equations give the exact value for the heat output; but the small coefficient of C_e indicates that K', i.e. the calorie value of 1 l. of expired air, is practically independent of the percentage of CO_2 in the expired air and, consequently, independent of the R.Q. As a result, the formulae can be simplified by using a mean value of C_e; but, since the variations in C_e are due partly to variations in the rate and depth of breathing and partly to variations in the R.Q., the residual error can be reduced to a minimum by expressing K' in terms of O_e and R.

Heat output in terms of percentage of oxygen in expired air and the R.Q.

One litre of expired air contains $O_e/100$ l. of oxygen. The corresponding volume of inspired air is $1 + O' - C' = 1 + (1 - R)\,O'$ l. and this contains $\{1 + (1 - R)\,O'\}\,O_i/100$ l. of oxygen. The difference is the volume of oxygen consumed, i.e. O' l. Therefore

$$\{1 + (1 - R)\,O'\}\,O_i/100 - O_e/100 = O';$$

from which we have $O' = (O_i - O_e)/\{100 - (1-R)\,O_i\}$,

or $O' = (O_i - O_e)/(79{\cdot}07 + 20{\cdot}93R)$.

Since the calorie output is equal to the volume of oxygen consumed multiplied by its calorie value per litre, we have from equation (7), omitting the protein correction,

$$K' = (O_i - O_e)\,(3{\cdot}941 + 1{\cdot}106R)/(79{\cdot}07 + 20{\cdot}93R).$$

For the range of R, 0·718–1·0, K' lies between $0{\cdot}05032\,(O_i - O_e)$ and $0{\cdot}05047\,(O_i - O_e)$, i.e. K' is practically independent of R and in fact with an error of less than 1 in 600 we can write

$$K' \doteq (O_i - O_e)\,0{\cdot}0504. \tag{17}$$

This equation is applicable when the protein correction is made from the urinary nitrogen. Since, despite its simplicity, it gives the same result as existing methods, the equation can also be used to compare new experimental data with other data in the literature in which the effect of protein metabolism has been neglected. If we use a mean value of O_e, e.g. 16·5, to adjust the coefficient of O_e the calculation can be done mentally. Thus

$$K' = (O_i - O_e)\,0{\cdot}0504 = 20{\cdot}93 \times 0{\cdot}0504 - 16{\cdot}5 \times 0{\cdot}0004 - 0{\cdot}050O_e,$$

i.e.

$$K' = 1{\cdot}048 - 0{\cdot}050O_e. \tag{18}$$

Including the protein correction, equation (17) becomes

$$K' = (O_i - O_e)\,0{\cdot}0504/(1 + 0{\cdot}082p). \tag{19}$$

If the percentage of calories from protein is $12\frac{1}{2}$, a typical mean value, K' can be read directly from the accompanying nomogram (Fig. 1) or from the simplified formula

$$K' = 1{\cdot}046 - 0{\cdot}050O_e. \tag{20}$$

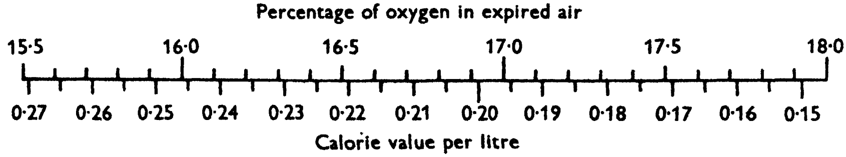

Fig. 1. From the percentage of oxygen in expired air, this nomogram gives the calorie value per litre of expired air. The heat output is given by multiplying the number of litres of expired air by the calorie value per litre. A protein correction is included on the assumption that 10–15 % of the total calories arise from protein metabolism.

A physiological curiosity is that when p is 10% equation (19) reduces to $K' = (O_i - O_e)/20$, i.e. the calorie output is five times the apparent oxygen usage.

Normal temperature and pressure and surface area nomograms

In routine calculations much time and labour can be saved by the use of nomograms. The N.T.P. nomogram (Fig. 2) gives the correction factor for reducing the observed volume of a gas saturated with water vapour to the volume measured dry at 0° C. and 760 mm. Hg.

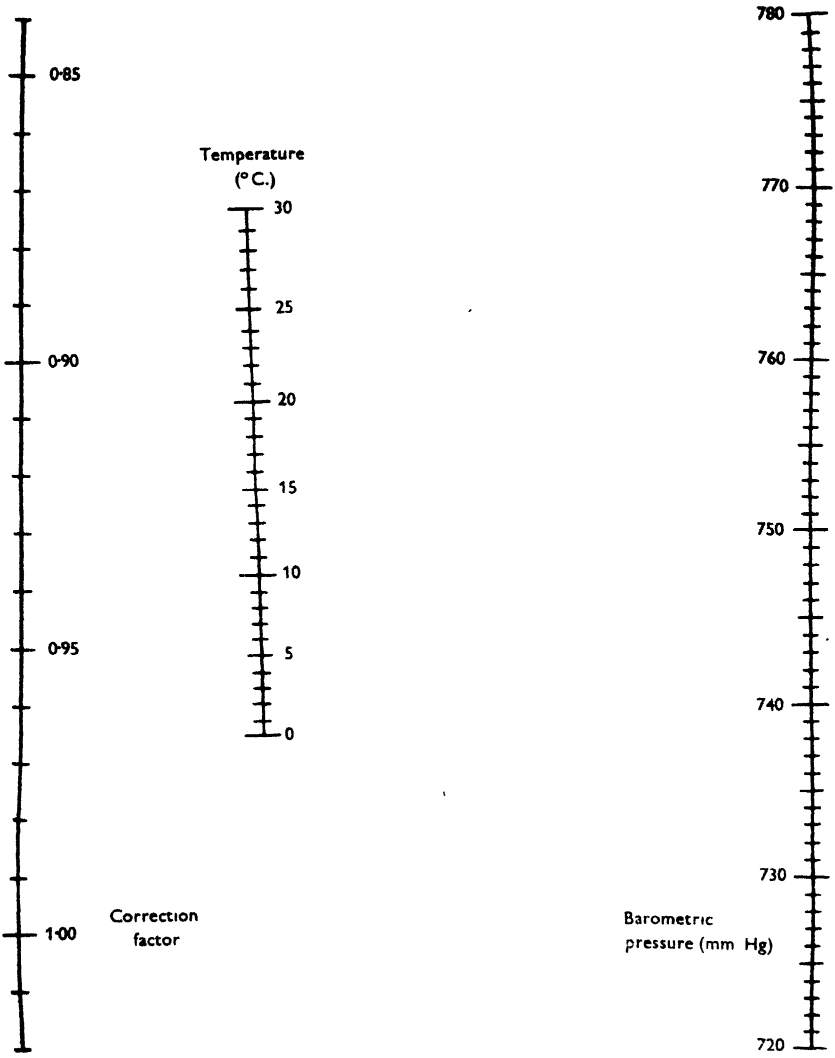

Fig. 2. This nomogram gives the correction factor for finding the dry volume at N.T.P. of a gas saturated with water vapour.

Existing surface area nomograms exemplify a dilemma. If they cover the possible range, the graduations are so crowded that accurate use is difficult or impossible. On the other hand, an open scale results in an inadequate range which is even more annoying. The nomogram of Fig. 3 evaluates the Du Bois formula

$$S = 0{\cdot}007184 \times H^{0{\cdot}725} \times W^{0{\cdot}425},$$

over a considerable range of height and weight, and the weight range can be extended if need be by entering the nomogram with half the observed weight and multiplying the area so found by $2^{0{\cdot}425}$, i.e. 1·343.

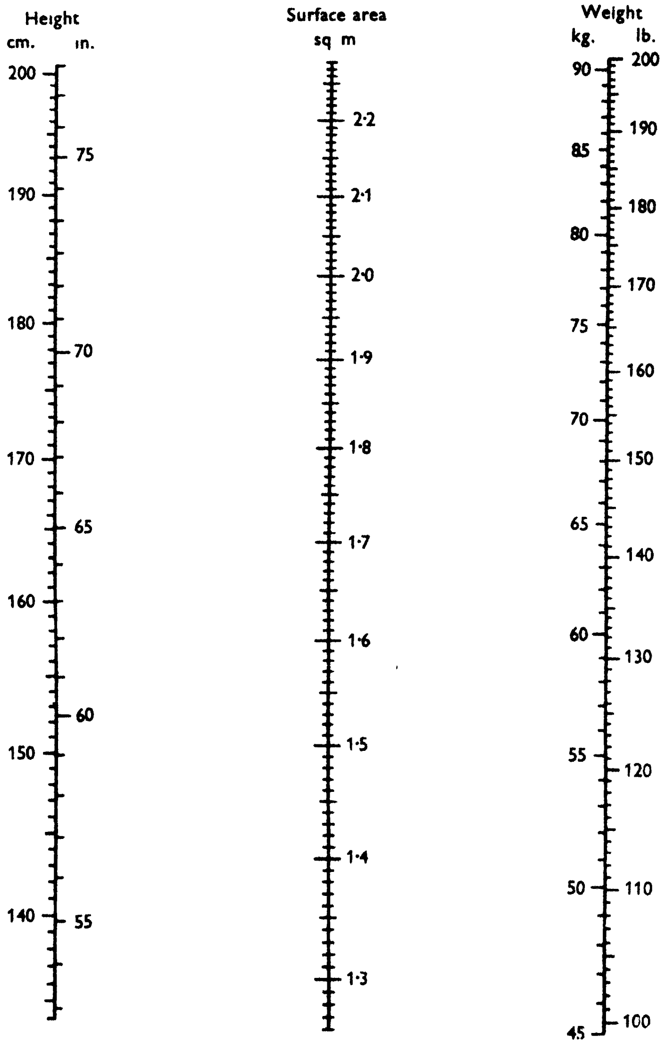

Fig. 3. Surface area nomogram. This evaluates the Du Bois formula. Both British and metric scales for height and weight are incorporated, and the weight range can be extended by entering the nomogram with half the observed weight and multiplying the resulting area by 1·343.

DISCUSSION

The numerical values in the formulae depend on the values of the fundamental data of Table 1. The general solutions are too complex for inclusion here; but other data such as the Zuntz and Schumburg figures for animal fat can readily be substituted.

The equations apply without modification to cases where anabolic processes are occurring, e.g. when fat is being laid down. Corrections similar to the protein correction can be made for other substances, e.g. the combustion of animal fat or of starch along with the deposition of human fat or of glycogen, and these indicate that such reactions should produce a negligible deviation from the present figures. The formula (10), heat output $= 3{\cdot}941 \times$ l. $O_2 + 1{\cdot}106 \times$ l. $CO_2 - 2{\cdot}17 \times$ g. urinary nitrogen, is in effect the theoretical partial regression equation of heat output on oxygen consumption, carbon dioxide production and urinary nitrogen. It shows the relative importance of these factors in indirect calorimetry. The corresponding experimental regression equation would give the closest agreement between direct and indirect calorimetry. It might well show, for example, that the Zuntz figure of 5·047 for the calorie value of oxygen in carbohydrate metabolism, which is very close to the value for starch, is somewhat too high for the post-absorptive state when, presumably, glucose or glycogen is the carbohydrate undergoing metabolism.

The employment of calorie values of oxygen based on the assumption that a fixed percentage of the total calories arise from protein metabolism was suggested by Magnus-Levy (1907). It is realized that for many purposes it is unnecessary and impracticable to make the 'exact' protein correction from the urinary nitrogen. A standard correction on the assumption that protein produces about 12½ % of the total calories has been suggested above. The error introduced, even if the percentage is somewhat different, is practically negligible. Widdowson (1947) has pointed out that the results of dietary surveys in various parts of the world all go to show that the majority of mankind take 10-15% of their calories in the form of protein. In various environments in different parts of the world Johnson & Kark (1947) found a range of 11–13%. It does not necessarily follow that there is a similar constancy in the percentage of calories arising from protein metabolism within the duration of any given metabolic determination; but Wishart (1928) found a marked parallelism between daily variations in basal metabolism and variations in the output of nitrogen in the urine.

The finding that the determination of metabolic rate requires only the measurement of the volume of air expired and its oxygen content introduces the possibility of new techniques. The first requirement is a simple, rapid and accurate method of measuring oxygen. Methods which depend on the paramagnetic properties of oxygen are being developed in several laboratories

(British Intelligence Objectives Sub-Committee, 1946; Pauling, Wood & Sturdivant, 1946; Rein, 1943) and already some forms of apparatus have sufficient accuracy to justify their use in metabolic experiments. Some instruments record automatically the oxygen content of a gas stream. This should allow the continuous measurement of metabolic rate over long periods.

In the meantime, however, the majority of laboratories will probably continue to rely on such well-established methods of indirect calorimetry as the Douglas-Haldane technique, and an example showing the simplicity of the calculation by the new method is given in the appendix.

SUMMARY

1. Equations are derived from first principles for the calorie value of a litre of oxygen metabolizing a mixture of carbohydrate, protein and fat. Simple methods for allowing for protein metabolism are described.

2. Heat output similarly can be expressed by such simple formulae as heat output (kg.cal.) $= 3{\cdot}9 \times$ litres oxygen consumed $+ 1{\cdot}1 \times$ litres carbon dioxide produced.

3. In ordinary breathing and in open methods of indirect calorimetry, the heat output is equal to the product of the volume of expired air (ventilation) and the calorie value per litre. This calorie value is almost exactly one-twentieth of the difference in the percentages of oxygen in inspired and expired air.

4. A surface area nomogram with special features and a nomogram for reducing the volume of gases to N.T.P., including the correction for aqueous vapour, complete an extremely simple method of computing metabolic rate.

It is a pleasure to record my indebtedness to Prof. E. P. Cathcart who has done so much in this field of physiology and to Prof. G. H. Bell and Prof. R. C. Garry for their advice and interest in these calculations.

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APPENDIX

Calculation of metabolic rate using Douglas bag method

The following data have been taken for comparative purposes from an article describing the Douglas bag method (Cathcart, 1918).

Volume of air expired in 10 min. 99·75 l., temperature of gas meter 16·8° C., atmospheric pressure 750 mm. Hg. Gas analysis of sample showed oxygen 16·86 % and carbon dioxide 3·54 %. Subject's height 163 cm., weight 50 kg.

From the second nomogram (Fig. 2) the factor for correcting the volume is 0·912 and therefore the volume of dry air at 0° C. and 760 mm. Hg is $99{\cdot}75 \times 0{\cdot}912 = 90{\cdot}97$ l.

From the nomogram of Fig. 1, the calorie value of expired air, including the correction for protein, is 0·203. The calorie output per hour is therefore $0{\cdot}203 \times 90{\cdot}97 \times 6 = 110{\cdot}8$ kg.cal. The surface area, Fig. 3, is 1·52 sq.m. and the metabolic rate is therefore $110{\cdot}8/1{\cdot}52 = 72{\cdot}9$ kg.cal./sq.m./hr.

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THE ACTION OF BOTULINUM TOXIN ON THE NEURO-MUSCULAR JUNCTION

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It has been known since the work of Edmunds and others (Edmunds & Long, 1923; Edmunds & Keiper, 1924; Dickson & Shevky, 1923*a*, *b*; Schübel, 1923; Bishop & Bronfenbrenner, 1936) that nearly all the phenomena of botulism could be explained primarily in terms of a peripheral action blocking somatic motor nerves and the parasympathetics. Guyton & MacDonald (1947) have recently confirmed and extended many of these observations in the intact animal. Modification of the factors involved in production of paralysis by the toxin can be more readily analysed in an isolated preparation.

We have found that if suitable concentrations of toxin are employed a rapid and reproducible neuro-muscular block can be produced in the isolated rat phrenic nerve-diaphragm preparation of Bülbring (1946). This preparation is adaptable and has proved very suitable for the analysis of the action of the toxin.

Torda & Wolff (1947) have claimed that choline acetylase is strongly inhibited by the toxin and that this might explain how the toxin produces neuromuscular block. The action of the toxin on this system and on the closely related aromatic amine acetylase has been investigated further.

METHODS

Toxin. Type A toxin was obtained from *Clostridium botulinum* (strain 4587) grown in a medium consisting of 25% tryptic digest of casein, 10% yeast extract, and 1% glucose. The ingredients were prepared as in CCY medium (Gladstone & Fildes, 1940). The toxin was purified by acid precipitation with HCl at pH 3·9, then rendered soluble by elution with 0·2M-Na_2HPO_4 to pH 6·0. The solution was freeze-dried and stored as a powder *in vacuo* over phosphorus pentoxide. As required, the toxin was dissolved in a buffer consisting of 1% Na_2HPO_4 and 0·2% gelatin at pH 6·6 to give a concentration of 2×10^5 or 2×10^6 mouse LD_{50}/ml. (20 g. mice, intraperitoneally). The toxin contained $1{\cdot}3 \times 10^7$ LD_{50}/mg. N. (Crystalline type A toxin contains $2{\cdot}4 \times 10^8$ LD_{50}/mg. (Lamanna, McElroy & Eklund, 1946), i.e. this material was c. 5% pure toxin.) In the text the amount of toxin used is expressed in terms of mouse LD_{50}/ml. of bath fluid which is for convenience called a unit.

Immunized rats. Rats were immunized by four fortnightly, intramuscular injections of alum-precipitated botulinum toxoid. Representative groups were subsequently shown to survive a challenge dose of toxin containing approximately 1×10^6 rat LD_{50}. Nerve-diaphragm preparations and antitoxic serum were obtained from this group of rats.

Rat diaphragm preparation. The preparation was as described by Bülbring; the bath fluid was Tyrode solution containing 0·2% glucose and aerated with 95% O_2, 5% CO_2. The bath volume was 100 ml. The phrenic nerve was stimulated through submersible electrodes (Brown, 1948) by slightly supramaximal rectangular pulses of 0·25 m.sec. duration delivered at a rate of 6/min. from an electronic stimulator. Either a torsion wire isometric lever or a spring-loaded lever was used to record the contraction. The preparation was always stimulated for ½–1 hr. before starting an experiment to allow stretching of the muscle to become complete and for the preparation to settle down to a steady contraction. In control experiments the diaphragm continued to contract for at least 3–4 hr. without appreciable decline.

Acetylcholine output. Two half diaphragms without their costal attachments were suspended in a 15 ml. bath in Tyrode solution containing 1 part/million eserine sulphate. After tetanizing at 50 cyc./sec. for 20 min. the fluid was pipetted off and assayed for acetylcholine by its depressor effect on the blood pressure of the chloralosed cat.

Acetylation of sulphanilamide. The enzyme was an acetone-dried powder of pigeon liver (Lipmann, 1945) ground with ice-cold KCl and phosphate buffer (pH 7·4). The supernatant was added to the rest of the system containing 0·02 M-$MgCl_2$, 0·02 M-sodium acetate, 0·05 M-NaF, 0·5 mg. ATP-7′-P and 0·12 mg. sulphanilamide, in a total volume of 3 ml. After incubation at 37° C. for 1 hr. the reaction was arrested with trichloroacetic acid and the free sulphanilamide estimated by Bratton & Marshall's (1939) method.

Choline acetylase. (*a*) The procedure of Torda & Wolff was followed except that in order to avoid loss of acetylcholine the solution was made acid with N/3 HCl before boiling to destroy the enzyme, and subsequently neutralized with N/3 NaOH. The yield of acetylcholine from minced mouse brain by this method is poor.

(*b*) The acetone dried rat brain powder system, as described by Feldberg & Vogt (1948), was used but without added activator.

The acetylcholine was assayed on the frog rectus abdominis muscle.

Choline esterase. Choline esterase activity of 1 ml. guinea-pig serum was estimated at 37° C. by Mendel & Rudney's (1943) method using acetylcholine, acetyl-β-methylcholine, and benzoylcholine as substrates.

RESULTS

Action of botulinum toxin on the rat diaphragm

After the addition of 1000–5000 units of botulinum toxin (Type A) to the bath fluid there is a latent period of 25–40 min. during which no effects of the toxin are visible on the tension record of the diaphragm to single motor nerve twitches. The tension then declines, at first gradually, then more rapidly and later again more slowly, so that the tension record shows a sigmoid form ending within 70–100 min. in complete paralysis of the diaphragm to nerve stimulation (Fig. 1). If the amount of toxin added to the bath is increased to 20,000–40,000 units the latency is only slightly decreased to 15–30 min. and the rate of paralysis somewhat accelerated. With 200–500 units the latency is increased in duration and the rate of paralysis is slowed; with smaller amounts still, no change is detected in preparations run for about 2 hr. 2000 units of toxin can be relied upon to produce paralysis regularly in a convenient time and this dose has been used for most of the experiments.

Toxin heated in a water-bath at 100° C. for 15 min. became inactivated, and lost its power to produce paralysis.

If the Tyrode containing the toxin was replaced by fresh Tyrode after the toxin had been in contact with the diaphragm for 5 min. or more, both the duration of the latent period and the rate of paralysis were unchanged. When washing was carried out during the first 5 min., however, the duration of the latent period was prolonged and the rate of paralysis slowed—an effect equivalent to reducing the amount of toxin added initially. It is evident that the toxin is 'fixed' by the preparation early in the latent period and that slowness of diffusion into the muscle is not responsible for the delay in the onset of paralysis.

The rat diaphragm was found to contract well over a wide range of temperature from 12 to 38° C. On cooling from 38° C. the tension at first increased somewhat, reached a maximum between 25 and 30° C. and then began to decline slightly; below 20° C. the speed of contraction was considerably decreased. In all experiments in which the preparation had been in contact with toxin at 17–18° C. for 2½ hr. no signs of paralysis of the diaphragm to nerve stimulation were observed (Fig. 2). If an intermediate temperature was employed, e.g. 28° C., both the latent period and the progress of paralysis were considerably prolonged. Corresponding results have been obtained in frogs (*Rana temporaria*). At laboratory temperature (16–18° C.) an injection into the dorsal lymph sac of 15,000 mouse LD_{50} of toxin/15 g. frog took 5–8 days to kill, whereas at 30° C. the same dose of toxin killed frogs in under 16 hr. It is thus evident that the Q_{10} of toxin action is high, resembling that of a chemical rather than a physical process. In the biological range the Q_{10} of diffusion is $<1{\cdot}1$ whereas that of chemical reaction is usually 1·5–3.

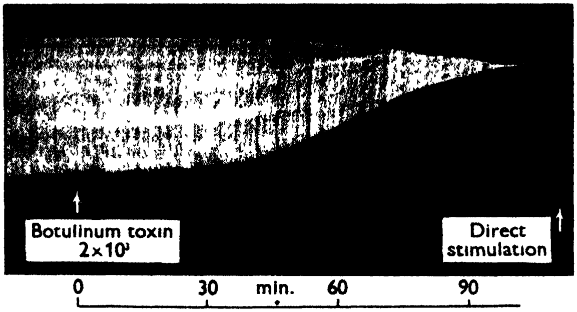

Fig. 1. Rat diaphragm preparation. Temp. 37·8° C. Botulinum toxin 2 × 10³ mouse LD_{50}/ml. added. Normal response to direct electrical stimulation.

Effect of Type B botulinum toxin. The rat phrenic nerve-diaphragm preparation is only slowly paralysed by 25,000 mouse LD_{50}/ml. of Type B botulinum toxin. These amounts are about 500 times greater than the amount of Type A toxin required to produce a similar rate of paralysis.

This great difference in the toxicity of Type A and Type B toxins was also seen in the whole rat, where the LD_{50} (intraperitoneal injection in 200 g. rats) of the two toxins were found to be approximately as follows:

$$\text{Rat } LD_{50} \text{ Type A toxin} = 25 \text{ mouse } LD_{50},$$

$$\text{Rat } LD_{50} \text{ Type B toxin} = 10{,}000 \text{ mouse } LD_{50}.$$

When, however, the phrenic nerve-diaphragm preparation was obtained from young guinea-pigs (150–200 g.) typical neuro-muscular block followed the addition of 2000 units/ml. of either Type A or Type B toxin. There were no marked differences in the latent period or rate of paralysis between Types A and B toxins on this preparation. The guinea-pig is known to be susceptible to Type B toxin.

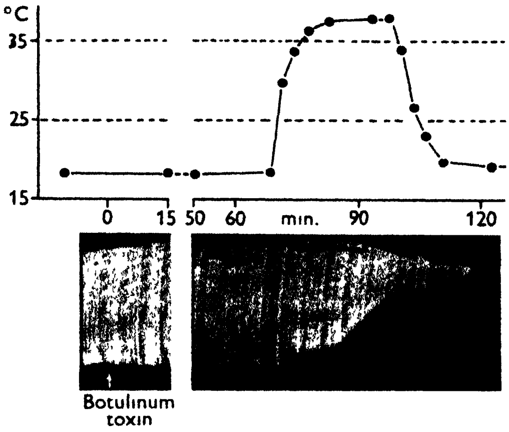

Fig. 2. Rat diaphragm preparation. Initially at 18·2° C. The graph shows the changes in temperature of the Tyrode solution during the course of the experiment.

Analysis of the paralysing action of botulinum toxin. Comparison with curare

The paralysis of the diaphragm to nerve stimulation is the result of neuromuscular block; the toxin does not affect the muscle fibres or the nerve trunk. As shown in the experiment of Fig. 1 the muscle responds normally to direct electrical stimulation when the paralysis to nerve stimulation is complete. To test the effect of toxin on the phrenic nerve trunk, a length of nerve together with the stimulating electrodes were immersed in a small glass cup of 1 ml. capacity not in communication with the main bath. The addition of 5000 units of toxin to the cup produced no change in the contraction of the muscle over the next 80 min., whereupon 2000 units of toxin were added to the main bath and paralysis ensued as usual 30 min. later.

Post-tetanic potentiation. Brown & v. Euler (1938) showed that in normal and partially curarized muscle, a short tetanus of the motor nerve increased the tension developed in the succeeding single twitches. Fig. 3 shows this phenomenon in the diaphragm where the effects of nerve stimulation at 6/min. are increased after a 5 sec. tetanus at 50 cyc./sec. After the addition of the toxin, the post-tetanic potentiation is tested at 10 min. intervals. The effect is unchanged during the latent period and during the paralysis the post-tetanic potentiation remains proportional to the twitch tension. As Brown & v. Euler (1938) showed that the post-tetanic potentiation was essentially a muscular phenomenon, and was present on direct stimulation of the fully curarized or denervated muscle, it is not surprising that it should be unchanged in botulinum poisoning and this fact offers further evidence of the lack of direct action on the muscle.

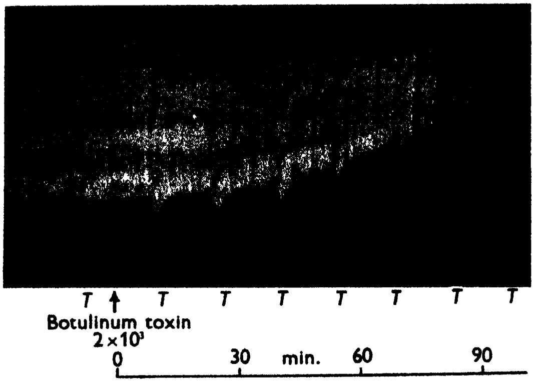

Fig. 3. Rat diaphragm preparation. Stimulated at 6/min. At points marked *T* rate of stimulation increased to 50 cyc./sec. for 5 sec. Botulinum toxin 2×10^3 units was added as indicated.

Tetani. When the phrenic nerve is tetanized at 50 cyc./sec. there is a rapid build-up of muscle tension in the first 5-10 sec. and the tetanus is then well maintained. The ratio of tetanus to single twitch tension is usually 2–3 : 1. After partial paralysis with toxin the response to tetanus remains perfectly normal in shape, but diminished in proportion to the decrease in twitch tension. Fig. 4 A, B, C shows 30 sec. tetani at different stages of botulinum intoxication. Fig. 4 D is from the same muscle before the addition of toxin, but when the twitch tension had been about 40% reduced by D-tubocurarine chloride 1 μg./ml. After an initial strong contraction which was about 50% greater than the twitch tension there was a rapid falling off of the tension.

Summation of two stimuli. When the summation curves of two successive stimuli applied to the phrenic nerve are examined (Fig. 5) it will be seen that the curve obtained with a muscle partly paralysed by toxin is very similar to the normal curve, showing only a small supernormal phase which is over in about 4 m.sec. In the partially curarized muscle, however, there is seen a typical large and prolonged supernormal phase due to the 'decurarizing' effect of the first stimulus (Eccles, Katz & Kuffler, 1941).

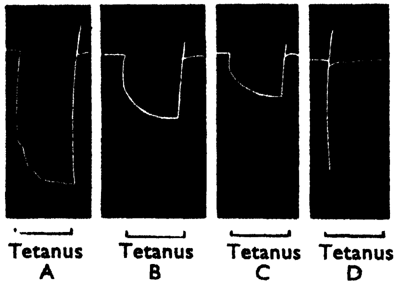

Fig. 4 A, B, C and D. Rat diaphragm preparation. Phrenic nerve stimulated at 50 cyc./sec. for 30 sec. A, B and C, stimulation at intervals as the course of the botulinum paralysis progressed. D, shows the response of the same preparation before addition of the toxin but when the twitch tension had been reduced to about 60% by 1 μg./ml. D-tubocurarine chloride.

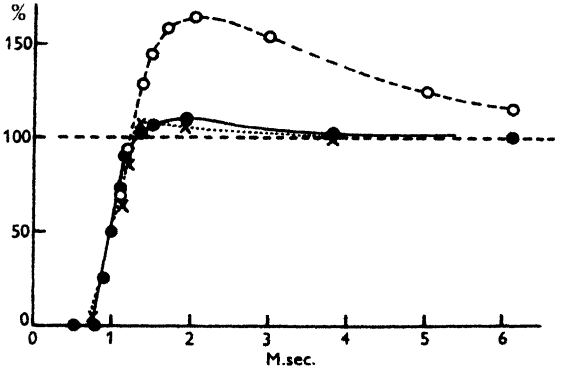

Fig. 5. Summation of two stimuli. Two stimuli delivered to the phrenic nerve from a Lucas pendulum and air-cored coils. Abscissa: interval between stimuli in m.sec. (approx.); ordinate: per cent increase in tension. ●—●, control preparation; O—O, partly curarized preparation; × · · · · ×, preparation poisoned with toxin.

Effects of drugs on the paralysis: eserine, prostigmine and *tetraethylpyrophosphate* (TEPP) were tested on the partially paralysed diaphragm. In low dosage (eserine 0·1–1 μg./ml.; prostigmine 0·01–0·1 μg./ml.; TEPP 0·01–0·1 μg./ml.) they produced a small, temporary increase of tension (Fig. 6*a*) which occasionally amounted to a maximum increase of 50% over the previous tension. This is similar to the degree of increase seen in the normal muscle. Larger amounts of prostigmine (1–5 μg./ml.) and TEPP (1 μg./ml.) produced paralysis in the poisoned diaphragm as in normal muscle. With curarized muscle, on the other hand, the small doses of all three drugs especially TEPP and prostigmine produced striking recoveries in the force of contraction of several hundred per cent, and the larger amounts of TEPP and prostigmine had no paralytic effect.

Potassium chloride added to the bath in concentrations of 2–15 mg./ml. produced in normal and poisoned preparations a small transient increase in tension followed by depression. In some poisoned preparations the initial transient augmentation was absent. This contrasts strikingly with the powerful anticurare action of KCl (Wilson & Wright, 1936).

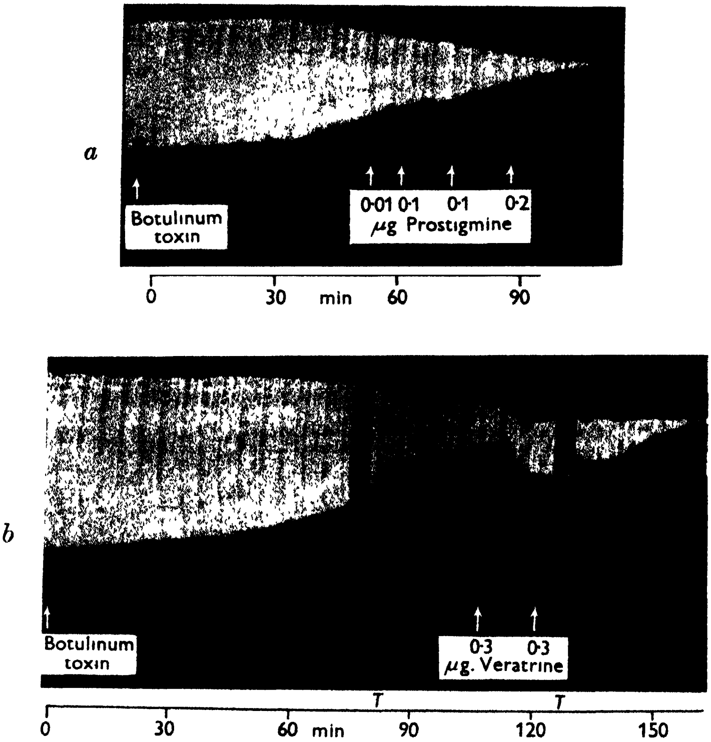

Fig. 6. Effects of drugs. Rat diaphragm preparation. Botulinum toxin 2×10^3 units. (*a*) Prostigmine 0·01, 0·1, 0·1, 0·2 μg./ml.; (*b*) veratrine 0·3–0·6 μg./ml. At *T*, *T* a maximal motor nerve tetanus at 100 cyc./sec., for 20 sec. was given.

Veratrine (mixed alkaloids) in suitable doses (0·1–1 μg./ml.) produced a striking increase in tension in normal muscle and also in that paralysed by toxin (Fig. 6 *b*). The twitch tension became comparable to that produced by a maximal motor nerve tetanus. Veratrine did not prevent ultimate paralysis.

Adrenaline (1–20 μg./ml.) occasionally produced a very small increase in tension in both the normal and poisoned preparation.

Close intravascular injections of acetylcholine. The neuro-muscular block produced by curare results from its action on the motor end-plates which become insensitive to the acetylcholine released from the motor nerve endings. During the neuro-muscular block caused by botulinum toxin, however, the motor end-plates remain sensitive to acetylcholine, as shown by the following experiments.

Acetylcholine, even in large amounts, will not reliably produce contraction of the diaphragm when added to the bath fluid. Close intravascular injections are, however, readily performed on the isolated rat diaphragm. The arterial supply is complex and unsuitable for this purpose, but practically the whole of each dome of the diaphragm drains into one large vein which empties into the inferior vena cava as it passes through the tendinous arch of the diaphragm (Fig. 7). Retrograde injection through the veins yields essentially the same results as close arterial injection.

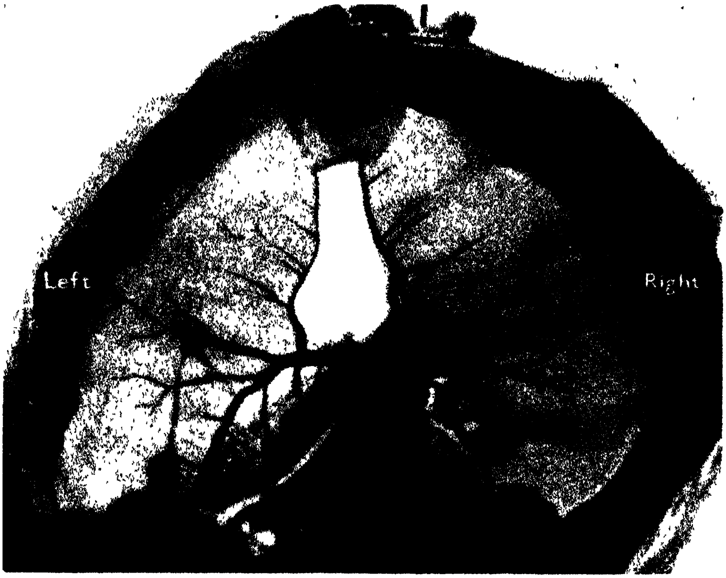

Fig. 7. Injected preparation of the venous drainage of the rat diaphragm. The large vein draining the left half of the diaphragm and the main right lateral vein can readily be seen.

The inferior vena cava is tied immediately above the entry of the hepatic veins and a needle cannula is inserted downwards into the thoracic inferior vena cava. The vein from the left side of the diaphragm is tied as it crosses the central tendon. A sector of the right diaphragm is cut on either side of the main lateral vein. The tendon end is then tied to a bar at the bottom of the bath and thread tied to the rib margin attached to the lever. Injections may be made or perfusion carried out through the cannula with only slight leakage of the injected fluid.

In Fig. 8 A the effect of injecting 10μg. acetylcholine is seen. A rapid twitch followed by a slight contracture is produced. Control injection of saline produced only very small mechanical effect. Fig. 8 B shows the response to acetylcholine of the same preparation when the tension due to motor-nerve stimulation had been reduced to about 10% by toxin and Fig. 8 C shows the acetylcholine response when the toxin had rendered motor-nerve stimulation completely ineffective. The response to 10 μg. acetylcholine is unaltered throughout the poisoning.

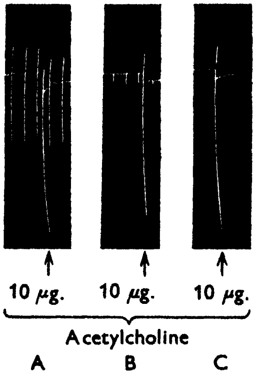

Fig. 8. Close intravenous injection of acetylcholine. A, normal preparation stimulated at 6/min. At the arrow 10 μg. acetylcholine were rapidly injected into the inferior vena cava. B, the same preparation after toxin when the response to nerve stimulation was reduced to 10–15% of the initial. C, the same preparation when it was unresponsive to nerve stimulation.

Liberation of acetylcholine on motor-nerve stimulation. Under favourable conditions the acetylcholine liberated in the diaphragm preparation by motor-nerve stimulation can be assayed. After the diaphragms had been contracting at 6/min. in Tyrode containing 1 part/million eserine sulphate the fluid was replaced twice. The nerve was then tetanized at 50 cyc./sec. for 20 min., and 30 sec. later the fluid was aspirated and assayed for acetylcholine. The nerve was then stimulated at 6/min. in fresh fluid for 15 min. and this procedure repeated. 5000 units of toxin were then added to the fluid bath. Seventy minutes later, when the preparation was almost completely paralysed, the stimulation and assay were repeated as before (Table 1). It will be seen that when the diaphragm preparation is paralysed by toxin the output of acetylcholine on motor-nerve stimulation is greatly reduced.

Table 1. Output of acetylcholine on stimulation of normal and poisoned rat diaphragm preparations

	Output of acetylcholine (μg./20 min.)	
	Before toxin	When paralysed by toxin
Exp. 1	0·125 0·120	<0·020
Exp. 2	0·105	<0·010

The immune diaphragm. Diaphragms from immune rats were set up in the usual way and carefully washed free of blood. Amounts of 2000 units of toxin never produced paralysis of the preparation. In one experiment a further 30,000 units were added an hour later, and after a further 75 min. still no paralysis had occurred. In another experiment 20,000 units of toxin were added at the beginning, and after 1 hr. a slow paralysis developed at a rate one might expect from the addition of about 200–300 units of toxin to a normal diaphragm. It seems most unlikely that the very small amount of occluded serum could account for this 100-fold increase in resistance to toxin. When diaphragms obtained from young guinea-pigs immunized to Types A and B toxin respectively were tested with both toxins, resistance was demonstrable only against the toxin type used to produce immunity.

Effect of antibotulinum serum. Homologous antibotulinum serum was used. When 1 ml. of serum was added to normal preparations 10 min. before the usual 2000 units of toxin, no paralysis ensued after 1 hr. A further 20,000 units of toxin, however, produced paralysis in 20 min. To see if the poisoned preparation could be restored by antitoxin 2000 units of toxin were added to the bath and 10 min. later the excess of toxin was washed out. Fifteen minutes later the usual paralysis made its appearance, and when it was half complete 2 ml. of antiserum were added. The paralysis proceeded at the usual rate and no recovery was observed during the next hour. Other experiments using high potency horse antibotulinum serum (100 antitoxin units/ml., i.e. sufficient to neutralize 10^6 units of toxin/ml.) were likewise unsuccessful in reversing botulinum paralysis.

Action of botulinum toxin on enzyme systems

Acetylation of choline. Botulinum toxin exerted no inhibitory effect on the acetylation of choline by either fresh or acetone dried rat brain.

TABLE 2. Choline acetylase activity of (*a*) minced, (*b*) acetone dried brain

Method *a*

	Acetylcholine synthesized (μg./g. wet brain/4 hr.)	
	Exp. 1	Exp. 2
Control	3·4 (2·9, 3·9)	5·5 (5·4, 5·6)
33 LD_{50} toxin/ml.	4·1 (3·4, 4·8)	7·7 (7·2, 8·3)
333 LD_{50} toxin/ml.	—	6·7 (6·6, 6·8)

Method *b*

	Acetylcholine synthesized (μg./g. acetone powder/hr.)	
	Exp. 1	Exp. 2
Control	204 (184, 224)	243 (235, 251)
20 LD_{50} toxin/ml.	208 (200, 216)	217 (204, 230)
200 LD_{50} toxin/ml.	—	240 (225, 255)

These results are not concordant with those of Torda & Wolff, who, however, were using a culture filtrate of unspecified purity as a source of toxin, and it is possible that some impurities present in their preparation may have been responsible for the inhibition of acetylcholine synthesis found in their experiments.

Acetylation of sulphanilamide. In ten control experiments, 56·7 % (S.E. ± 0·7) of the added sulphanilamide was acetylated. Botulinum toxin produced no decrease in the acetylation of sulphanilamide, whereas 0·02 M-hydroxylamine produced its usual powerful inhibition (Table 3).

TABLE 3. Acetylation of sulphanilamide

	Acetylation % of control
Toxin 66 LD_{50}/ml.	96 (97, 97, 94)
Toxin 666 LD_{50}/ml.	96 (97, 95)
Toxin 6666 LD_{50}/ml.	96 (95, 97)
Hydroxylamine 0·02 M	8 (11, 5)

Choline esterase. Botulinum toxin even in very high concentrations has no effect on the 'true' or 'pseudo' choline esterase of guinea-pig serum. The absence of inhibitory effect is not due to a lag period, as when the toxin-enzyme mixture was incubated at 37° for 1 hr. before addition of substrate, there was still no appreciable effect on choline esterase activity (Table 4).

TABLE 4. Choline esterase activity with and without addition of botulinum toxin

	µl. CO_2/ml. serum/10 min.		
	Acetyl choline	Acetyl-β-methyl-choline	Benzoyl choline
Serum alone	293·8	23·2	124·5
Serum + 40,000 LD_{50} toxin/ml.	280·8	24·2	123·5
Serum + 40,000 LD_{50} toxin/ml.; activity tested 1 hr. later	—	25·5	—

Metabolism of rat brain slices. 100 units toxin/ml. had no significant effect *in vitro* on the Q_{O_2} of rat brain slices determined in the Warburg apparatus as compared with controls.

160 LD_{50}/g. wt. were injected intraperitoneally into a rat. After 4½ hr. the animal was moribund, its brain was removed and the Q_{O_2} determined on slices. Again there was no significant difference from the normal values.

DISCUSSION

Botulinum toxin produces paralysis of the diaphragm preparation after a characteristic delay which is related to the size of dose given. This delay is typical of tetanus, diphtheria, and other toxins, and Pappenheimer (1947) has shown for tetanus toxin that the duration of the latent period before signs appear in the intact animal is roughly inversely proportional to the logarithm of the dose of toxin. That this delay in the case of botulinum toxin is not due to diffusion of a large molecule has been clearly shown here, since replacing with fresh Tyrode during the latent period—a procedure which reverses the diffusion gradient into a muscle—does not prevent or alter the course of the subsequent paralysis. During the latter part of this phase the toxin is fixed and presumably producing some change at the neuro-muscular junction which ultimately leads to block. This process is unlikely to be purely physical as the duration of the latent period and the rate of paralysis are much prolonged by lowering the temperature, the process having a high Q_{10} which is characteristic of a chemical reaction.

The irreversible paralysis produced by botulinum toxin has been shown to result from a neuro-muscular block leaving muscle and nerve trunk unaffected. The mechanism by which the toxin produces the block is entirely different from the block produced by curare. The normal type of tetanic response, the normal summation curves, and the lack of response to anticurare drugs suggest that the partially poisoned preparation contains some motor units that behave normally in all ways, and blocked motor units in which transmission is not restored by conditioning stimuli or other means.

The site of the block has been localized. The end-plate is normally sensitive to acetylcholine but the output of acetylcholine from the nerve endings is greatly diminished, whereas curare renders the end-plates insensitive to acetylcholine without decreasing its release from the motor-nerve endings (Dale, Feldberg & Vogt, 1936). The diminished acetylcholine output can be conceived as occurring in the following possible ways: (1) Transmission of the nerve impulse through the fine unmyelinated nerve fibres to the 'acetylcholine release region' in apposition to the end-plates may be blocked; in this case *no* acetylcholine will be released at the affected neuro-muscular junctions. (2) The synthesis of acetylcholine by the nerve endings is inhibited; since all or none inhibition of enzymes has not been described, in this case it would be expected that the power to synthesize acetylcholine would gradually decline, until the amount of acetylcholine fell below the threshold needed to activate the end-plate. (3) The release of acetylcholine may be decreased, perhaps by a permeability change in the nerve endings; a change of this nature is also unlikely to be all or none.

A diminished acetylcholine output due to increased choline esterase activity and hence greater breakdown can be excluded, since the output of acetylcholine was measured in the presence of eserine, a choline esterase inhibitor, and because of the lack of effect of toxin on choline esterase *in vitro.*

If mechanism (1) were correct, the effect of anticholinesterase drugs would be similar on a partly paralysed preparation to that on the normal preparation, i.e. the production of a brief tetanic contraction in response to a single nerve impulse in those muscle fibres whose neuro-muscular junctions were still unaffected by toxin; at the blocked neuromuscular junctions the anticholinesterase will be ineffective since acetylcholine is not being released at all and hence cannot be preserved by choline esterase inhibition. On the other hand, if explanation (2) were correct, the amount of acetylcholine released at the blocked neuro-muscular junctions has fallen below threshold and might be raised above threshold by inhibition of choline esterase and conduction restored in at least a proportion of the blocked junctions. Together with the normal effect on the unpoisoned junctions, the resultant increase in force of contraction would be more marked than we have seen in botulinum poisoning. *In vitro*, contrary to the results of Torda & Wolff, we have found no inhibitory effect on choline acetylase in minced mouse brain, nor on the far more active system utilizing an acetone dried powder of rat brain. Since Lipmann & Kaplan (1946) have shown the liver enzyme system acetylating aromatic amines has the same coenzyme (coenzyme A) as choline acetylase, we tested the effect of toxin on this system without finding any inhibitory effect. Explanation (3) seems to us unlikely, but cannot confidently be excluded owing to lack of knowledge of the final mechanism of acetylcholine release.

While it is not yet possible to localize with certainty the site of action of botulinum toxin, the evidence presented could most readily be explained by an interaction of toxin with the fine unmyelinated nerve fibres entering the end-plate region, thus causing a block in the transmission of the nerve impulse.

Botulinum type A toxin has been crystallized and is now known to be a globular protein of mol. wt. 900,000–1,100,000 (Putnam, Lamanna & Sharp, 1946). A complete amino-acid analysis (Buehler, Schantz & Lamanna, 1947) of the toxin has revealed no abnormal amino-acids or suggestion of abnormal peptide links or any considerable amount of constituents other than amino-acids. These analytical results make the problem of how this toxin produces its effects very obscure. Its high molecular weight makes it most improbable that it could enter a cell at all readily, and yet the very minute amounts of toxin required are rapidly and selectively taken up by the neuro-muscular region. Since the ultimate mechanism of action of the toxin is at present so obscure, the present demonstration of specific toxic action on an isolated preparation should prove of value in facilitating further investigation.

The degree of insensitivity of the rat diaphragm preparation to Type B toxin as compared with Type A toxin correlated well with the insensitivity of the whole rat to Type B toxin, and would suggest that the differing sensitivity of other animals to toxin is related to the sensitivity of their motor-nerve terminals rather than to differing modes of distribution or disposal of the toxin. The results with guinea-pig diaphragm illustrate this point since this diaphragm, like the intact guinea-pig, does not show a marked discrimination for either toxin.

The resistance of the preparation obtained from rats actively immunized with toxoid is of great interest. These results cannot be explained as due to occluded serum antitoxin, since an amount of serum from the same animals equal to about three times the volume of the diaphragm failed to give the same protection. It may be that the antitoxic activity of the whole preparation is greater than that of an equivalent amount of serum, but the possibility also exists that this resistance may be a tissue immunity induced by inoculation that renders the motor-nerve terminals highly resistant to the toxin. A good deal of further work will be needed before the exact mechanism involved can be decided. Lastly, the lack of reversal by antitoxin was not unexpected in view of the results of Zamecnik & Lipmann (1947) with *Cl. welchii* lecithinase. These authors found that once this enzyme was in contact with substrate, subsequent addition of antitoxin, except in massive amounts, exerted only a minor antagonistic effect.

The experimental findings discussed here are concordant with the symptoms and signs of clinical botulism. Guyton & MacDonald found that after local botulism of the gastrocnemius muscle of guinea-pigs, partial recovery of function occurred only very slowly over the following year. The lack of action of antiserum on the paralysed preparation is in agreement with the observation that antiserum is ineffective in reversing clinical botulism.

Note added in proof. We have now shown that results identical with those described above are given by an electrophoretically homogeneous toxin of $180 \times 10^6 LD_{50}$/mg. N. Since this paper was submitted similar conclusions have been reported from experiments on the intact animal by Ambache (1949).

SUMMARY

1. Botulinum toxin produces irreversible paralysis of the isolated rat phrenic nerve-diaphragm preparation after a latent period.

2. Boiled toxin does not produce paralysis.

3. Removal of the toxin during the latent period does not prevent the paralysis.

4. The latent period is greatly prolonged and the rate of paralysis slowed by lowering the temperature.

5. Prior addition of antibotulinum serum will prevent the development of paralysis by the toxin, but addition of antitoxin subsequent to toxin will not affect the paralysis.

6. Diaphragms from immune rats are highly resistant to botulinum toxin.

7. Some 500 times larger doses of Type B botulinum toxin than of Type A toxin are required to paralyse the rat phrenic nerve-diaphragm preparation; this correlates with the larger LD_{50} of Type B toxin in the rat. Both the whole guinea-pig and the guinea-pig phrenic nerve-diaphragm preparation are sensitive to both Type A and Type B toxins.

8. The paralysis produced by botulinum toxin on the rat phrenic nerve-diaphragm preparation is due to neuro-muscular block. Conduction in the nerve is unaffected and the muscle responds normally to direct stimulation.

9. The neuro-muscular block produced by toxin differs from that of curare in that motor-nerve tetani are well maintained, the summation curve for two stimuli is normal and that the paralysis is unaffected by eserine, prostigmine, TEPP, potassium or adrenaline.

10. The neuro-muscular block produced by the toxin differs from that of curare in that the motor end-plates remain sensitive to acetylcholine and in that the output of acetylcholine on motor-nerve stimulation is greatly reduced.

11. A method is described by which close intravascular injections of acetylcholine can be made into the diaphragm preparation by retrograde injections through a cannula tied into the vein.

12. The toxin does not affect the enzymes acetylating choline and sulphanilamide, nor choline esterase.

13. It is assumed that any effect of the toxin on the motor nerve must occur after it has lost its medullary sheath on entering the end-plate. By an irreversible fixation of the toxin to the fine nerve fibres, transmission of the impulse through them is abolished, consequently no release of acetylcholine takes place, resulting in the observed neuro-muscular block.

We are indebted to Dr G. L. Brown, Dr D. W. Henderson and Dr C. A. Keele for helpful criticism. The preparation and partial purification of the toxin as well as the mouse-assays and the immunization of rats were carried out for us by Dr J. Keppie in the Microbiological Research Department, Porton.

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THE DEVELOPMENT OF SENSITIVITY TO ACETYLCHOLINE IN DENERVATED MUSCLE

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It is known that denervated muscle responds to doses of acetylcholine much smaller than those to which it reacts in its normal state. How the change in sensitivity develops in relation to time, however, does not appear to have been closely studied (see Tower, 1939). Brown (1937) observed, in the gastrocnemius of one cat, that the threshold dose of acetylcholine 4 days after denervation was no less than half that for normal muscle, and the 'contracture' was just visible. Six days after denervation the high sensitivity and the slow phase of the response were 'fully developed'. One of us (Reid, 1942), observing the change in response of the cat's gastrocnemius to acetylcholine from 1 to 6 days after denervation, found that contractures occurred as early as the third day, but that a high degree of sensitivity was not present until the fifth day, at which time fibrillation also first appeared.

This paper records a more detailed study of the change in the effect of acetylcholine, using the technique of close arterial injection described by Brown (1938).

METHODS

Observations were made on the tibialis anterior muscles of twenty-eight cats on the first eight, the tenth, fifteenth, twenty-third, twenty-fourth and twenty-fifth days after the muscle had been denervated either by crushing the lateral popliteal or by cutting the sciatic nerve aseptically. On the selected day the cat was decerebrated under ether, after injection of 1–2 mg. atropine sulphate subcutaneously, and the tibialis anterior was prepared for close arterial injection. Control observations were made in the opposite limb. Each injection was 0·2 c.c. in volume, and consisted of the appropriate dilution in saline, prepared immediately before injection, of a 1/1000 solution of acetyl choline in acid saline prepared the same morning. Since it is known (Brown, 1937) that the speed of injection affects the response, all injections were made as rapidly as possible. A Brown-Schuster isometric myograph was used. The muscle was kept moist by a saline drip.

Between injections the muscle was stimulated to give single maximal twitches by break shocks from an induction coil, applied by leads attached to the tendon and the femoral drill. The smallest

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('threshold') dose of acetylcholine was first determined to which the muscle would respond with a contraction unmistakably greater than that elicited by saline alone. The response of the muscle was then recorded on a fast drum to doses of 1, 5 and 20 μg. acetylcholine chloride.

RESULTS

The threshold dose. In the controls, and in the denervated muscles before the onset of sensitivity, the threshold was sharp (Fig. 1, upper panel), and its determination presented less difficulty than that of the denervated sensitive muscles. In all except two it was between 0·1 and 1·0 μg., and the threshold of these muscles differed from the nul dose by not more than 0·1 μg. That is to say, a muscle with a threshold of 0·3 μg. just responds to 0·3 μg. and does not respond to 0·2 μg. In Fig. 2 are shown the results of twenty-one control threshold determinations in innervated muscles. They approximate to a normal distribution about a mean. The arithmetic mean is 0·35 μg.

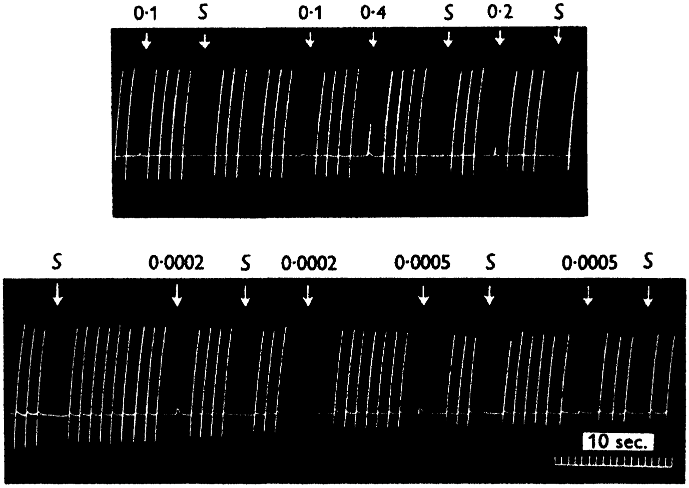

Fig. 1. Upper panel. Determination of the 'threshold' dose of acetylcholine required to cause a contraction in an innervated tibialis anterior muscle of a cat. The unlettered responses are single maximal twitches. Close intra-arterial injections were given at the arrows, *S* denoting saline, and the figures μg. of acetylcholine. The lower panel shows the responses of the corresponding muscle of the other leg, denervated 7 days previously.

In the denervated sensitive muscles one difficulty encountered was that the preparation became less sensitive as more injections were given. In the lower panel of Fig. 1 it can be seen that the first dose of acetylcholine (0·0002 μg.) elicited a small but definite response, which differed from a response of corresponding magnitude in an innervated muscle by its greater duration. However, a second dose of the same size, freshly prepared, was without effect, though the muscle responded to 0·0005 μg.; a further injection of 0·0005 μg. provoked a smaller response. It was our custom, therefore, to start with a dose smaller than the smallest recorded threshold (0·00005 μg.) and increase each succeeding dose twofold until a response was obtained greater than that given to saline alone.

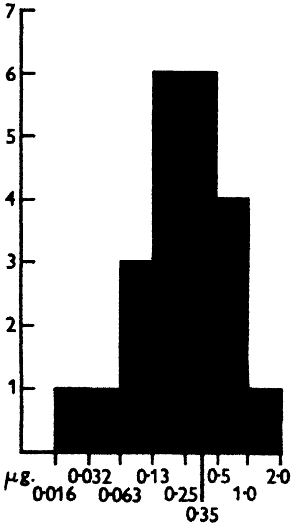

Fig. 2. Frequency distribution of threshold doses of acetylcholine required to cause contractions in control innervated muscles. Ordinates: number of cats; abscissae: threshold dose in μg. (logarithmic scale).

A second difficulty was that some muscles did respond slightly to saline. When this occurred the threshold was taken as that dose of acetylcholine which caused a contraction unequivocally greater than this 'saline' contraction. The latter was thought to be due to a physical expansion of the muscle when the injection was made, since it occurred, when present, on injection of Ringer or plasma as well. It was seldom encountered in the later preparations when great care was taken to avoid any constriction or obstruction to venous drainage.

There was no correlation between the sensitivities of an innervated muscle and its corresponding denervated fellow. For example, one muscle, 10 days after denervation, responded to 0·0005 μg. and its particularly sensitive control to 0·02 μg.; another more sensitive muscle, on the twenty-fourth day after denervation responded to 0·0001 μg. and its particularly insensitive control to 1·0 μg. Nor was there any correlation between sensitivity and weight. The mean threshold of muscles 8 or more days after denervation was 0·00035 μg.

In Fig. 3 are depicted the thresholds determined in both innervated (circles) and denervated muscles (dots). For the first 4 days there is no significant increase in sensitivity. On the fifth day two out of three muscles are no more sensitive; the third is twelve times as sensitive as its control. After the eighth day all the muscles, with one exception, are several hundred times more sensitive than their controls; the exception is fifty times as sensitive. It appears, therefore, that the change in the muscle which renders it sensitive to acetylcholine becomes manifest quite sharply on the sixth and seventh days after denervation. Neuromuscular transmission was absent from the third day onwards.

The onset of fibrillation. We were surprised to find that fibrillation could not be seen in the tibialis anterior as late as 6 days after denervation, whereas it was clearly visible in the gastrocnemius and soleus on the fifth day. On the sixth day fibrillation was present in the tibialis anterior in only one out of four experiments, but in this case as in five experiments on the seventh and eighth days it could be seen only in isolated patches and mainly on the under surface. Usually it was more readily seen in the lower part of the neighbouring extensor digitorum and in the peroneal muscles. In one animal on the tenth day fibrillation was as vigorous in the tibialis anterior as in the gastrocnemius, and in the other animal it was scarcely more active than on the seventh and eighth days.

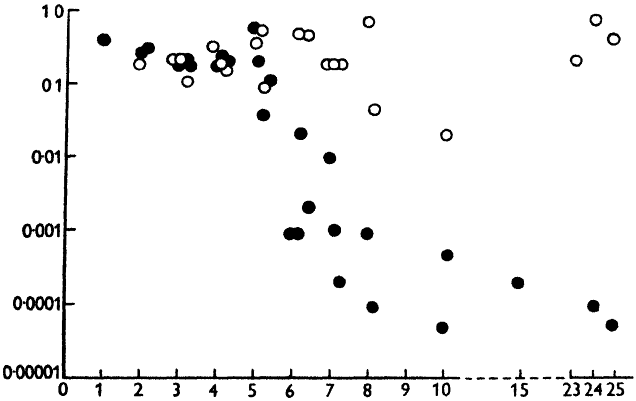

Fig. 3. Threshold doses of acetylcholine required to cause contractions in innervated and denervated muscles. Ordinates: threshold dose in μg. (logarithmic scale); abscissae: number of days since denervation. Dots: threshold of denervated muscles. Circles: threshold of corresponding innervated muscles.

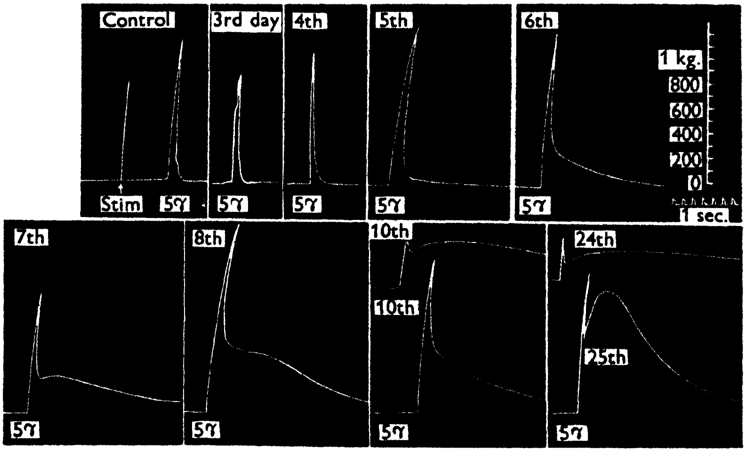

Fig. 4. Development of contractures in response of denervated muscles to close intra-arterial injections of 5 μg. acetylcholine. First panel: a single maximal twitch (drawn stationary) and the response to 5 μg. acetylcholine of an innervated control. Succeeding panels: responses of muscles denervated for increasingly long periods.

It was, however, vigorous in the animals examined on the twenty-fourth and twenty-fifth days.

The development of the 'slow component'. In Fig. 4 are shown the responses of the tibialis anterior to close arterial injection of 5·0 μg. acetylcholine 3, 4, 5, 6, 7, 8, 10, 24 and 25 days after denervation. The upper left-hand panel shows a single maximal twitch (on a stationary drum) and the response to 5 μg. acetylcholine of the innervated muscle corresponding to that whose response 5 days after denervation is shown three panels further on. In each of the last two panels records from two different muscles are shown.

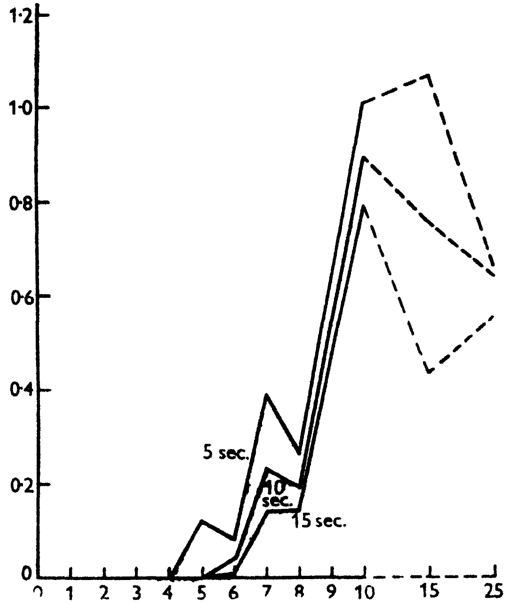

Fig. 5. Development of the 'slow component' of the contractions of denervated muscles in response to close arterial injection of acetylcholine. Ordinates: ratio to the initial twitch tension, developed in response to 5 μg. acetylcholine, of the tension maintained at 5, 10 and 15 sec. after the injection. Abscissae: number of days since denervation.

It can be seen that the response to 5 μg. of acetylcholine on and after the fifth day consists of an initial twitch and a prolonged maintenance of tension—the 'slow component'. When the height of the tension still maintained at 5, 10 or 15 sec. is expressed as a fraction of the initial twitch tension, it is found (Fig. 5) that this ratio increases rapidly from the fifth day onwards. With larger doses (20 μg.) short-lived contractures lasting up to 10 or 15 sec. could be seen as early as the third day. Thus the slow component makes its appearance earlier than the increase in sensitivity to acetylcholine, and continues to increase relative to the twitch after maximum sensitivity has been reached.

DISCUSSION

These results show that the sensitivity of the tibialis anterior to close arterial injections of acetylcholine does not increase progressively from the time of denervation or of cessation of neuro-muscular transmission, but becomes manifest on the sixth and seventh days. This relatively sudden and delayed appearance of sensitivity invites curiosity about its cause. One hypothesis has been that the sensitivity is due to a loss of cholinesterase. Early estimations (Martini & Torda, 1937), (Couteaux & Nachmansohn, 1938) of the cholinesterase content of denervated muscle failed to distinguish between the so-called 'true' and 'pseudo-' cholinesterase. The classification of the family of cholinesterases and their relation to the function of different tissues is still controversial (Bodansky, 1946), but it can be said that there is so far insufficient evidence to suggest that the concentration of any cholinesterase alters in a manner parallel to the development of sensitivity during the period following denervation.

Another, more widely held, hypothesis is that the sensitivity not only to acetylcholine but also to potassium, nicotine and caffeine (Dale & Gasser, 1926; Kuffler, 1943) is attributable to membrane changes (see Tower, 1939). If, however, such changes develop progressively from the time neuromuscular transmission ceases, one would expect also a gradual increase in sensitivity to injected doses of acetylcholine from the third day onwards. It is possible, therefore, that after neuromuscular transmission ceases, some process continues which is not itself directly related to the stimulation of a muscle by acetylcholine, but which must proceed to a critical stage before sensitivity can be demonstrated.

Although no muscle was found to be fibrillating, which was not already sensitive to acetylcholine, some sensitive muscles fibrillated feebly or not at all. These results therefore neither support nor exclude the possibility that fibrillation is due to the action of acetylcholine on a sensitive muscle. Of more interest in this respect is the earlier observation (Reid, 1942) that curare greatly reduces the response of denervated muscle to acetylcholine while it continues to fibrillate.

SUMMARY

1. Observations were made by the method of close arterial injection, into the tibialis anterior of the cat of the 'threshold' dose of acetylcholine required to cause a contraction in innervated muscles, and in muscles denervated 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 23, 24 and 25 days previously.

2. It was found that sensitivity to acetylcholine develops quite rapidly on the sixth and seventh days after denervation. The slow maintained tension in

response to larger doses of acetylcholine appears earlier, but continues to develop relative to the twitch after sensitivity is established.

3. Fibrillation takes longer to develop and is less marked in the tibialis anterior than in the gastrocnemius. Some muscles which were sensitive to acetylcholine were fibrillating feebly or not at all.

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CHANGES IN IONIC DISTRIBUTION FOLLOWING DIALYSIS OF AQUEOUS HUMOUR AGAINST PLASMA

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There have been frequent attempts to determine, by studies of ionic distribution, whether the aqueous humour and blood plasma are in thermodynamic equilibrium. It has been customary to adopt, as a criterion of equilibrium, the theoretical distribution of monovalent ions calculated by Van Slyke (1926) from the base-binding power of the plasma proteins. This distribution corresponds to a ratio: *concentration of sodium in plasma/concentration of sodium in aqueous humour* (R_{Na}) equal to 1·04, and to a similar ratio for the chloride ion, R_{Cl}, equal to 0·96. Studies on the cat certainly suggest a close approximation to thermodynamic equilibrium between the two fluids. However, a study of the chloride distribution in the dog (Hodgson, 1938; Davson & Weld, 1941) revealed a considerable discrepancy from Van Slyke's theoretical ratio, a mean value for R_{Cl} of 0·92–0·93 being obtained; the distribution of sodium, on the other hand, was almost completely theoretical ($R_{Na} = 1·04$). There thus seems to be an 'excess' of chloride in the aqueous humour of the dog and, to a less extent, in that of the cat. Studies on dialysates of plasma, obtained with collodion sacs (Ingraham, Lombard & Visscher, 1933; Greene & Power, 1931), suggest, however, that Van Slyke's theoretical ratios of 1·04 and 0·96 are wrong in that they fail to take into account the possibility that the activity coefficients of the ions are not the same for the two fluids; the studies indicate that R_{Na} should be 1·08 or even higher, whilst R_{Cl} should be 0·98–0·99. If this is true, the ratios of 1·03 and 0·945 for sodium and chloride in the cat, for example, are not to be taken as evidence of approximate thermodynamic equilibrium between the two fluids but rather of the existence of an excess of sodium and chloride in the aqueous humour. If aqueous humour and blood plasma are not in thermodynamic equilibrium in respect to sodium and chloride, dialysis of the one fluid against the other should result in the passage of these ions across the separating membrane. If, moreover, our interpretation of the values of R_{Na} and R_{Cl} is correct, the passage should be from the aqueous humour into the blood plasma. In the experiments to be described, such movements have been consistently found.

METHODS

General. Dogs and cats were anaesthetized with nembutal; aqueous humour was removed from both eyes with a needle and syringe; blood was obtained by heart-puncture, heparinized, and equilibrated with alveolar air at body temperature; the plasma was separated by centrifugation in a corked tube. For studies on chloride or conductivity the fluids from a single animal were sufficient for an experiment; for studies on the distribution of sodium, the fluids from three animals were pooled in strict proportion.

Dialysis. In experiments nos. 1–12, the plasma was placed in a collodion sac, prepared as described by Dale & Evans (1920), tied to the end of a piece of capillary tubing (Fig. 1*a*), which was then sealed with sealing-wax. The sac was washed with a few drops of aqueous humour and then immersed in sufficient of this fluid to cover it. A collar of rubber tubing round the capillary tubing permitted the sac to stand in the fluid without touching the sides or bottom of the container. Evaporation was reduced (but apparently not always completely prevented) by covering the collar with paraffin-wax. The system was allowed to stand for about 16 hr. at room temperature, together with samples of the original fluids in stoppered tubes. During the course of the work it became evident that, in some of the experiments, evaporation of the fluids in the dialysis system was taking place, consequently the apparatus illustrated in Fig. 1*b* was devised. It consisted of an outer glass container, *A*, into which fitted a ground stopper, *B*. The base of the stopper formed

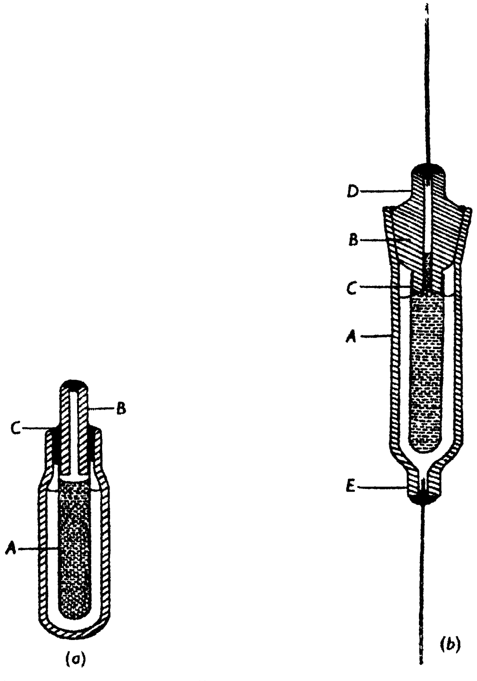

Fig. 1. (*a*) Simple dialysis system. The collodion sac, *A*, is tied to a piece of capillary tubing, *B*, and submerged in aqueous humour. *C* is a rubber collar. (*b*) Completely sealed system. For description see text.

a slightly tapered projection, *C*, which was pushed into the top of the collodion sac, the junction being sealed with cellulose acetate paint. The stopper and the bottom of the container were pierced by holes, into each of which was sealed, by means of sealing wax, a length of glass tubing drawn out to capillary thinness as shown in the diagram. The stopper with its attached collodion membrane was placed in the container and fixed in position by wax or paint spread on the outside junction. An outer projection on the stopper, *D*, was pushed into a hole in a rubber bung; into the other end of this hole was pushed a glass tube joined to a length of rubber tubing so that by sucking or blowing on to this tubing the collodion sac could be collapsed or blown out. To fill the chamber, the sac was blown out, the bottom capillary placed under the required solution which was then drawn in by suction until the sac was completely collapsed. The chamber was then removed from the bung, inverted, and another similar projection, *E*, on the bottom of the container pushed into the hole. The sac was then filled with the other solution, normally plasma, by sucking it in through the capillary in the stopper. Some suction was maintained on the tube until the tip of this capillary was sealed with wax. The container was then removed from the bung and the other capillary likewise sealed.

By this means equal volumes of fluid were obtained inside and outside the sac. The sac was made 8 mm. in diameter and the inside of the container about 11 mm.; if the sac was cut so that it almost touched the bottom of the container, the fluid levels inside and outside were about the same. For the experiments described here two chambers were constructed to take 1 or 2 ml. of solution inside the sac, but it is not of course necessary, though it is desirable, that the chamber should be completely filled, either inside the sac or outside.

Stirring was achieved by fixing the chamber on a large pulley which was made to rotate at about 1 rev./sec.; at this speed the air bubbles in the compartments moved through the whole column of liquid and back during each cycle. Preliminary studies showed that equilibrium was established in about 30 min., but generally 2–3 hr. were allowed.

To empty the sac the tips of the capillaries were broken off and the filling procedure followed in reverse. Even with the operations required in the repeated washing of the sac before filling, it should remain serviceable for at least half a dozen dialyses; it must, of course, be kept wet when not in use.

Chemical. Sodium was determined by the Barber-Kolthoff (1928) technique on triplicate 0·5 ml. samples of the fluids ashed as described earlier (Davson, 1939). The coefficient of variation of means of triplicate determinations was estimated to be 0·23 % (thirty-four sets of determinations); since the effects described in this paper involve changes of as much as 1–2 % there is no doubt of their statistical significance. Chloride was determined on 0·2 ml. samples by the Sendroy technique (1937); the coefficient of variation of means of triplicates was 0·6 % (twenty-nine sets of determinations).

Dry weight. About 1 ml. of plasma was evaporated to dryness on a sand-bath and heated for about 24 hr. in an oven at 105°.

Conductivity. The conductivity cell was in the form of a pipette of capacity 0·3 ml. with a flattened bulb into which were sealed two platinum wire electrodes, blackened in the usual manner. Connexion to the bridge was made by two copper wires soldered on to the electrodes and carried round the stem of the pipette. The junctions and leads were protected with a coating of wax and lacquer. The bulb was filled by suction through a rubber tube which was then clipped off and the tip of the pipette was sealed with a length of blocked cycle-valve tubing. The cell was immersed in melting ice contained in a Dewar flask. Before filling with a sample the cell could either be washed with distilled water and dried with alcohol and ether or merely washed out with a fluid of conductivity close to that expected in the sample. The resistances were measured on a bridge constructed of normal radio components and calibrated with a decade resistance box. Duplicate readings on plasma and aqueous humour samples agreed to one part in a thousand.

pH. The measurements were made with a glass-electrode against a calomel half-cell on a direct-reading valve millivoltmeter. Alternate measurements were made on dialysed and undialysed fluids which were transferred as rapidly as possible to the measuring cell so as to avoid serious losses of CO_2.

RESULTS

Chloride. The results with chloride on dogs are quite definite (Table 1); as a result of a decrease in A, the concentration of chloride in the aqueous humour, and of a rise in P, the corresponding concentration in the plasma, the ratio, R_{Cl}, changes from a mean value of 0·91 to one of 0·98. The two fluids are quite clearly not in equilibrium with respect to chloride; moreover, Van Slyke's calculated figure of 0·96 for the ratio differs significantly from the experimentally determined ratio after dialysis, 0·98. In cats the effects are not so large, as we should expect since the average value of R_{Cl} is, on the basis of earlier work, normally 0·945; nevertheless, there is a definite change in the ratio following dialysis when this is low in the original fluids. It would appear that in some animals the fluids are in equilibrium in respect to chloride whilst in others they are not. It will be noted from Table 1 that the decrease in concentration in the aqueous humour does not always equal the increase in plasma concentration; this results from several causes. Thus the volumes of the two fluids were not always equal; evaporation occurred in some experiments, and this tended to obscure the loss of chloride from the aqueous humour; however, even when these effects were excluded by the use of the new type of sac (Exps. 17–24), the rise in concentration in the plasma was on occasions larger than the fall in the aqueous humour.

TABLE 1. The change in the Gibbs-Donnan ratio, R_{Cl}, following dialysis of plasma against aqueous humour

Exp.	A_1	P_1	R_1	A_2	P_2	R_2	ΔR
1	138·4	121·2	**0·875**	130·3	126·0	**0·965**	+0·09
2	145·4	130·0	**0·895**	140·1	134·6	**0·960**	+0·065
3	142·2	127·1	**0·895**	133·1	133·2	**1·000**	+0·105
4	134·5	123·9	**0·920**	129·0	129·9	**1·007**	+0·087
5	127·7	119·2	**0·935**	125·0	122·0	**0·975**	+0·04
6	131·7	123·0	**0·935**	132·1	128·5	**0·975**	+0·04
7	130·2	123·8	**0·950**	131·3	130·7	**0·995**	+0·045
17	128·0	118·2	**0·923**	126·9	123·0	**0·970**	+0·047
18	123·2	114·4	**0·930**	122·1	118·5	**0·972**	+0·042
19	129·9	115·9	**0·893**	121·6	122·4	**1·005**	+0·112
20	127·5	109·9	**0·865**	122·5	115·2	**0·940**	+0·075
Mean			**0·911**			**0·979**	
21	131·6	122·7	**0·932**	127·5	126·0	**0·990**	+0·058
22	127·5	123·9	**0·973**	128·9	124·6	**0·968**	−0·005
23	130·6	119·1	**0·910**	129·5	123·4	**0·953**	+0·043
24	132·3	127·7	**0·965**	132·0	128·4	**0·973**	+0·008
Mean			**0·945**			**0·971**	

Exps. 21–24 refer to cats, the remainder to dogs. A and P are the concentrations of chloride, in millimoles per kg. of water, in aqueous humour and plasma respectively. The suffixes (1) and (2) refer to the figures obtained before and after dialysis respectively.

Sodium. The results with this ion are shown in Table 2; they are equally consistent but not so striking in magnitude. The average value of R_{Na} changes from 1·036 to 1·057 in the dog, and from 1·042 to 1·068 in the cat. Once again we find that the change in aqueous humour concentration is not always balanced by that in the plasma. In Exps. 8 and 9 the sac had been maintained in distilled water before use, a circumstance which produced a diluting effect on the system and thus obscured the rise in plasma concentrations; in all succeeding experiments the sac was equilibrated with aqueous NaCl, the concentration being approximately that found in the aqueous humour. There was, however, evidence of evaporation, an effect which tended to obscure the fall in concentration of the aqueous humour (Exps. 10–12); this is well brought out by Exp. 12, in which sufficient fluids were obtained for two successive dialyses. As a result of the first dialysis the value of R_{Na} rose from 1·028 to 1·066; new lots of the same fluids were then placed inside and outside the sac and the system left for another 16 hr., whereupon the value of R_{Na} did not differ significantly from that obtained after the first dialysis, although as a result of evaporation the concentrations of sodium in aqueous humour and plasma both rose. In the later experiments (nos. 13–16) evaporation was excluded by the use of the new type of chamber; R_{Na} invariably increased as a result of dialysis, as before, but even under these conditions the loss in concentration in the aqueous humour was not entirely accounted for by a rise in the plasma concentration. It is difficult to account for these discrepancies; it is possible that the collodion membrane itself is not in equilibrium with the fluids surrounding it; it may tend to swell, to adsorb or desorb ions, the particular effect observed depending on its previous history. In view of the large number of animals required and the extreme laboriousness of the chemical technique, it was not thought worthwhile to investigate the point further. The significant fact is that all ten

TABLE 2. The change in the Gibbs-Donnan ratio, R_{Na}, following dialysis of plasma against aqueous humour

Exp.	A_1	P_1	R_1	A_2	P_2	R_2	ΔR
8	150·4	154·6	**1·027**	146·6	155·6	**1·062**	+0·035
9	150·3	155·0	**1·032**	147·4	154·7	**1·050**	+0·018
10	149·8	157 2	**1·050**	149·1	160·5	**1·077**	+0·027
11	148·5	155·8	**1·049**	149·1	158·7	**1·064**	+0·015
14	149·8	154·0	**1·028**	148·5	154·1	**1·038**	+0·01
16	147·4	152·1	**1·032**	145·7	152·8	**1·049**	+0·017
Mean			**1·036**			**1·057**	
12	155·8	160·2	**1·028**	156·6	166·9	**1·066**	+0·038
	155·8	160·2	**1·028**	158·5	168·2	**1·061**	+0·033
13	152·3	160·8	**1·056**	150 3	161·2	**1·073**	+0·016
15	152·1	158·7	**1·043**	149·3	159·3	**1·067**	+0·024
Mean			**1·042**			**1·068**	

Exps. 12, 13 and 15 refer to cats, the remainder to dogs. Pooled specimens from three animals used in each experiment. *A* and *P* are the concentrations of sodium, in millimoles per kg. of water, in aqueous humour and plasma respectively. The suffixes (1) and (2) refer to the figures obtained before and after dialysis respectively.

dialyses, representing the fluid from twenty-seven animals, concurred in showing that the ratio R_{Na} increased as a result of dialysis; dilution or concentration of the system by a few per cent should produce slight errors in the ratio, but these would be too small for detection (cf. for example Exp. 12).

Conductivity. The changes in conductivity are shown in Table 3. In all but one case there was an increase in the resistance of the aqueous humour and a decrease in that of the plasma, i.e. the changes were such as could be produced by a movement of sodium and chloride from the aqueous humour into the plasma. It may be computed, from the characteristics of the dialysis system, that the ratio: decrease in resistance of plasma/increase in resistance of aqueous humour should be in the region of 1·4; the ratio of the mean deviations was found to be 1·5.

TABLE 3. Changes in electrical resistance of plasma (Δr_{Pl}) and aqueous humour (Δr_{Aq}) as a result of dialysis

Exp.	Δr_{Aq}	Δr_{Pl}	$-\frac{\Delta r_{\mathrm{Pl}}}{\Delta r_{\mathrm{Aq}}}$
25	1·7	−3·6	2·1
26	2·5	−8·7	3·5
27	0·6	−0·7	1·2
28	1·8	−2·1	1·2
29	1·0	−1·8	1·8
30	1·2	−2·6	1·45
31	7·3	−10·6	1·45
32	0·5	−1·7	2·4
33	−0·1	−1·2	−12·0
34	6·5	−4·3	1·5

Exps. 25–33 on cats; Exp. 34 on a dog. In Exps. 30 and 32, whole blood was placed in the sac instead of plasma.

pH. The increase in the value of R_{Na} might conceivably be due to an alkalinity of the dialysis system resulting from the loss of CO_2; the observed changes, however, would require an increase of about 50% in the base-binding power of the plasma—a change that could be achieved only by an increase of pH in the region of one unit (Van Slyke, Hastings, Hiller & Sendroy, 1928). It was not feasible to make pH measurements on the fluids used for analysis; separate dialysis experiments were therefore carried out with both types of sac to determine whether any significant change in pH of the two fluids took place as a result of the dialysis. It was found, in general, that the pH of the plasma was unaffected by dialysis, whilst the aqueous humour tended to become more acid; presumably this is because the losses of CO_2 in the manipulation caused the aqueous humour to become alkaline; dialysis with the better buffered plasma tended to restore the pH to the physiological range.

The re-formed aqueous humour. Earlier work (Davson & Weld, 1941) has shown that the rapidly re-formed aqueous humour exhibits a value of R_{Cl} of about 0·96, i.e. it approaches that for a dialysate. In the present work the fluid, re-formed within about half an hour of withdrawal of the normal aqueous humour, was analysed for sodium; the results are shown in Table 4. It will be observed that R_{Na} has closely approached unity, i.e. it has shifted farther away from the correct figure for a dialysate of plasma instead of nearer to this figure as was found with the chloride ion. The protein concentration in the re-formed fluid was of the order of 3% so that we may expect a lower value of R_{Na} at equilibrium, i.e. 1·03–1·035 instead of 1·06–1·07; the observed value, however, is much less than this.

TABLE 4. The Gibbs-Donnan ratio in the re-formed aqueous humour

Exp.	R_1	R_2	R_3	Protein in re-formed aqueous humour (%)
9	1·032	1·050	1·012	—
10	1·050	1·077	1·010	3
11	1·049	1·064	1·024	2·5
12	1·028	1·064	1·011	3

R_1 and R_2 are the ratios obtained with the normal aqueous humour before and after dialysis respectively; R_3 is the ratio obtained with normal plasma and re-formed aqueous humour.

DISCUSSION

The results show that the aqueous humour is not in thermodynamic equilibrium with blood plasma in so far as the distribution of sodium and chloride between the two fluids is concerned. In view of the presence of a metabolizing unit (the lens) within the eye, of the fact that arterial plasma has been arbitrarily chosen as the reference fluid, and of the circulation of the aqueous humour through the eye, this is perhaps not surprising. Nevertheless, the quantitative aspects of the results point strongly to the existence of a secretory mechanism for the maintenance of a high concentration of sodium and chloride in the aqueous humour. Thus the high concentration of sodium in the aqueous humour might possibly be accounted for on the basis of a difference of pH between plasma and aqueous humour resulting from the metabolism of the lens; the difference in pH required to produce the observed value of R_{Na} would be too small to detect with any certainty. Any explanation of the excess of sodium on the basis of a difference of pH between aqueous humour and plasma would require, however, that the chloride concentration in the aqueous humour should be below that necessary for equilibrium. The results have shown, however, that the excess of chloride in the dog's aqueous humour actually exceeds that of sodium. Again, the concentrations of sodium in arterial and venous plasma are not appreciably different, so that it is unlikely that the concentration in the capillary plasma—the fluid with which the comparison should be made—differs measurably from that in the arterial plasma used. Finally, although the fact that the aqueous humour is continually drained off and replaced might account for small deviations from diffusion equilibrium, it cannot account for the high concentration of sodium and chloride in the aqueous humour: the osmotic forces brought into play are too great to be accounted for by the available filtration pressure.

We have seen that the rapidly re-formed aqueous humour shows a value of R_{Na} of about 1·01. The fluid formed under these conditions generally contains some 3% of protein. A simple exudate of plasma with this protein content should show a value of R_{Na} of about 1·030–1·035 and the observed value of 1·01 could be due to a differential leakage of albumin, to a filtration too rapid to permit of the attainment of a Donnan equilibrium, or to a secretory process. There is at present no evidence, derived from other observations on the re-formed fluid, to indicate which of these explanations is the most likely.

In the dog, the deviation of R_{Cl} from equilibrium was considerably greater than that of R_{Na}, the excess of chloride in the aqueous humour amounting to about 7%, or some 9 m.mol./l.; since the discrepancy of sodium amounted to only about 3 m.mol./l., the results suggest that some other cation is present in excess; studies on the distribution of potassium (Davson, Duke-Elder & Benham, 1936), calcium and magnesium (Stary & Winternitz, 1932) have not revealed excesses of these ions in the aqueous humour of a magnitude sufficient to account for the excess of chloride. Presumably an organic cation is present. In the cat the deviation of the chloride ratio from the equilibrium value was considerably smaller, and on occasions it was found that the chloride ion was actually at equilibrium; in this respect, therefore, there seems to be a significant difference in physiology between the cat and dog.

To conclude, the present work demonstrates beyond all reasonable doubt that secretory activity is concerned in the formation of the aqueous humour; whether the fluid is to be regarded purely as a product elaborated by the epithelial cells of the ciliary body or as a filtrate from plasma with its concentration modified by contact with these epithelial cells, cannot yet be decided.

SUMMARY

The concentrations of sodium and chloride were determined in the aqueous humour and plasma of cats and dogs before and after dialysis against each other by means of a collodion sac. As a result of this dialysis the distribution of these two ions changed in the sense that both sodium and chloride diffused from aqueous humour to plasma. A study of the changes in electrical conductivity of the fluids following dialysis confirmed these findings. It is concluded that the two fluids are not in thermodynamic equilibrium and that the deviations are of such magnitude and sign as to warrant the assumption of secretory activity.

We are indebted to the Medical Research Council for defraying the whole expenses of this research and for a personal grant to one of us (D.M.M.). The chloride determinations were carried out by Miss P. A. Matchett.

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A METHOD FOR THE DETERMINATION OF HEPARIN IN BLOOD

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Best (1946) has pointed out that an accurate method for the determination of small amounts of heparin in blood is very desirable for many studies in the field of blood clotting and thrombosis. The methods available for this purpose up to the present have been the correlation of heparin concentration with the clotting time (cf. Jaques, 1939), the titration of heparin with protamine (cf. Jaques & Waters, 1941), and the isolation of the heparin by the procedure of Charles & Scott (1933). The first method is applicable only to a very limited range of concentrations and requires that there be no change in factors affecting the clotting system other than heparin. The protamine titration also requires a clotting system and is of little value with small concentrations of blood heparin. The Charles & Scott procedure can be applied only to large volumes of blood containing high concentrations of heparin. For these and other reasons, experiments have been undertaken in this laboratory in an attempt to develop a direct method for blood-heparin estimations.

The method for the recovery of heparin from blood described in this paper is an application of the fact that the strongly acid heparin will form salts with proteins or complex bases in stoichiometric proportions (Fischer & Astrup, 1935; Fischer, 1935; Jaques, 1943). In a private communication, Dr A. F. Charles of the Connaught Medical Research Laboratories, University of Toronto, informed us that heparin combines with the straight-chain amine, *N*-octylamine. On the basis of his suggestion, tests were made of this reagent and we found that *N*-octylamine hydrochloride reacted with heparin at the pH of the blood, and also precipitated heparin directly from plasma. Furthermore, a critical concentration of octylamine precipitated over 90% of heparin added to plasma with a minimal precipitation of protein. The stable octylamine salt of heparin could then be hydrolysed by heating to 70° C. in relatively strong (0·1 N)-NaOH, and the opaque, colloidal solution obtained could be purified by precipitating the heparin with brucine at pH 5·5–6·0.

METHODS

A. *The extraction of heparin from blood*

Reagents

Sodium citrate: 3·8 % solution.

Octylamine: 7 % (v/v) solution. To 7 ml. of *N*-octylamine add 7 ml. of distilled water and 3·4 ml. of concentrated hydrochloric acid. After the octylamine has gone into solution add enough distilled water to make up to a volume of 100 ml. The pH is usually between 7·0 and 8·5. If above or below this range, the pH must be adjusted by the cautious addition of concentrated hydrochloric acid or NaOH. A fresh octylamine solution must be made weekly.

0·1 N-NaOH *and* 0·05 N-NaOH.

Ether.

Ethyl alcohol 95 %.

Brucine phosphate solution: 10 g. of brucine alkaloid are dissolved in 50 ml. of warm 95 % ethyl alcohol. Concentrated phosphoric acid (Merck, sp.gr. 1·750) is diluted 1 : 10 with distilled water and 14 ml. of the dilute acid are added to the warm brucine solution. The solution is set aside to evaporate to dryness. The crystalline brucine phosphate is obtained as white needles; 5 g. of brucine phosphate are dissolved in 100 ml. of water. The pH of this solution must be 5·5.

Procedure

Nine ml. of blood are drawn and added to 1·0 ml. of sodium citrate solution and centrifuged. To the plasma, 1·0 ml. of the octylamine solution is added, and after standing 2 min., the mixture is centrifuged at 2000 r.p.m. for 10 min. The supernatant is decanted and the tube allowed to drain several minutes. The precipitate is broken up thoroughly with a stirring rod and 2·0 ml. of 0·1 N-NaOH are then added and the tube placed in a water-bath at 70° C. for 15 min. The tube is cooled to 30–40° C., 4·0 ml. of ether are added and the octylamine extracted from the aqueous layer by inverting the tube gently three or four times. The ether layer is removed with a pipette and the remainder of the ether removed by placing the tube in a water-bath at 40° C., the temperature of which is gradually raised to 70° C.

The tube is then cooled and 4 ml. of the brucine phosphate solution are added. After standing 5 min., the precipitate of brucine-heparin is collected by centrifuging at 2000 r.p.m. for 5–10 min. The supernatant is then decanted off and the tube allowed to drain. The precipitate is washed by mixing with 4·0 ml. of 95 % ethyl alcohol, using a stirring rod. After centrifuging, the precipitate is similarly washed with 4·0 ml. of ether and centrifuged, the ether is decanted off, and the remaining ether is removed by drying the precipitate in a vacuum desiccator for at least 1 hr.

To the precipitate are added 2·0 ml. of 0·05 N-NaOH solution and the tube is placed in the water-bath at 70° C. for 15 min. It is then cooled to room temperature.

The heparin present in this extract can now be estimated by one of the known methods, after adjusting the pH by the addition of buffer. The method we have used routinely for the determination of heparin is the estimation of its metachromatic activity with Azure A as described by Jaques, Mitford & Ricker (1947). We have also used the thrombin method described by Jaques & Charles (1941), particularly for low concentrations of heparin in blood.

Remarks on the procedure

Citrate must be used as the anticoagulant, since octylamine precipitates with oxalate and in fact will clot oxalated blood. The amount of octylamine used appears to be sufficient to precipitate the heparin quantitatively and also to precipitate a small portion of the plasma proteins. A larger quantity of octylamine precipitates increasing amounts of protein. The small quantity of protein precipitated with this quantity of octylamine appears necessary to ensure satisfactory flocculation of the octylamine-heparin precipitate. With an occasional sample, it is necessary to add slightly more octylamine to obtain satisfactory separation of the precipitate. This is observed particularly in fatty human plasmas. On standing 30 min., the octylamine may cause a gelling of the plasma and hence it is necessary to centrifuge immediately. For hydrolysis of the octylamine-heparin compound, a temperature of 70° C. is required and it is advisable to use a constant temperature water-bath. After hydrolysis, an opaque, colloidal solution is obtained. The opacity clears on warming after treatment with ether but reappears when all the ether has been driven off.

Under the conditions described (a brucine phosphate concentration of 3·3% and a pH of 5·5–6·0), brucine precipitates heparin quantitatively. Washing with alcohol and ether removes not only excess brucine but also lipids and colour which interfere with the determination of heparin. If much colour is present as a result of haemolysis this can be removed by longer contact with the alcohol.

After hydrolysis of the brucine-heparin compound, a clear solution is usually obtained. Rarely, a slight protein precipitate appears and may give a weak gel. It is then necessary to add the buffer, used in the assay procedure presently to be described, to the tube in order to provide sufficient volume of solution that the protein can be removed by centrifuging.

B. *The determination of heparin in blood extracts*

The method used routinely by us is the method of Jaques *et al.* (1947) based on the measurement of the metachromatic activity of heparin with the dye, Azure A. Certain minor modifications to increase the sensitivity of this method have been made and are included in the following description. For a discussion of the principles involved in this method and sources of error (interfering substances, etc.), reference should be made to the original paper.

Reagents and equipment

Azure A: 100 mg. % of Azure A (certified biological stain of dye content 82%). This is diluted 1 : 8·5 before use.

Phosphate buffer: a potassium phosphate buffer, pH 7·3 and 0·15M-phosphate is prepared. This is in place of the borate buffer used by Jaques *et al.* The latter is not suitable for use when the heparin is to be determined at the same time by its antithrombin activity. If other methods are used for the final determination of the heparin, the buffer may need corresponding modification.

Lovibond tintometer: model no. 1. The slide carrying the neutral tint glasses is replaced with a slide carrying each of 10 and 20 red, 10 and 20 blue glasses. To increase the length of light path without a marked increase in volume of the solution, a specially constructed cell (inside dimensions 9 mm. wide, 27 mm. long, and 38·5 mm. high) is used. The light apertures of the tintometer box are partially masked to reduce the width to less than that of the glass cell (9 mm.).

Procedure

Unknown heparin solutions are dissolved in saline or distilled water as desired. No measurable metachromatic difference has been observed whether the heparin is dissolved in saline or in distilled water. Saline is used if an assay for anticoagulant activity is to be conducted on the same solution.

To a given volume of the heparin solution is added saline to 2·0 ml. Two ml. of buffer are added

and then 1·0 ml. of Azure A solution. The solution is mixed and transferred to the glass cell. The colour of the cell solution is matched immediately in a Lovibond tintometer using the red and blue glasses. From the red reading obtained, the heparin content of the sample is determined on the standard curve (Fig. 1). For maximum accuracy, a dilution which will give a red reading between 5 and 10 is found for the final assay. With unknown heparin solutions of high concentrations, the dilution required for this may be determined approximately by diluting the first assay with an equivalent dilution of the dye in buffer and saline until the red reading falls into the desired range. As recommended by Jaques *et al.*, instead of a duplicate determination, a second reading is made with a quantity of the unknown less or greater than that used in the first determination.

The calibration curve for the Lovibond tintometer is established using a series of dilutions of the standard heparin solution. Some difference in the values for the calibration curve are found with different observers but the values are reproducible for any given observer.

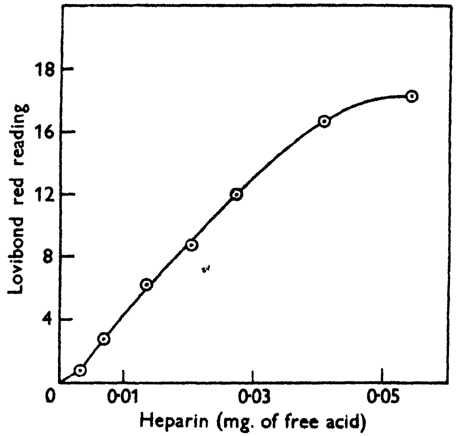

Fig. 1. Calibration curve for the estimation of heparin metachromatically.

Expression of heparin concentration

The concentration of heparin in blood and tissues, in agreement with general biochemical usage, should be expressed in terms of the weight of heparin in a given volume or weight of the material. Up to the present there have been relatively few investigations on heparin where this was a problem. While heparin is issued commercially on the basis of anticoagulant units, it would appear advisable to reserve this for expressions of the anticoagulant activity, *per se*. Heparin concentration might be expressed in milligrams of equivalent barium salt, sodium salt, or of the free heparin acid. The use of the barium salt of Charles & Scott as the reference expression has the advantage of ease of convertibility from weight to anticoagulant units (1 mg. = 100 units). The sodium salt will be the actual comparison standard used in most cases. Both these salts are produced *in vitro* and do not represent the actual heparin present, probably in the form of a protein salt. In agreement with the custom used for many biologically active substances we express the concentration of heparin as mg. of free acid heparin. The value reported by Jaques & Charles (1941) for the anticoagulant potency of beef heparin is 156 units/mg. of free acid heparin present.

In all cases, we determine the concentration of heparin in terms of equivalent weight of the Provisional International Standard of heparin, issued by the National Institute for Medical Research, London, for the Health Organization of the League of Nations. In the absence of figures for the salt content of the International Standard, it is not possible to determine directly the amount of free acid heparin present. However, from sulphur content is obtained a conversion factor of 0·884 for converting the weight of International Standard to weight of free acid heparin. It is to be understood then that all values will be reported as 0·88 × equivalent weight of International Standard. While the factor used may be in error by as much as 3 % and is subject to future correction, this error is not significant for the purposes of the method. This represents an assigned value of 148 units per mg. for the anticoagulant activity of the free acid heparin in the International Standard.

Heparin standard

As standard, a sample was used of the Provisional International Standard of heparin (130 i.u./mg.) supplied through the courtesy of Dr F. C. MacIntosh. A solution of 76·9 μg. (10·0 i.u./ml.) was prepared, and with this the standard curve (Fig. 1) has been constructed. For routine standard solutions, a secondary standard prepared from Connaught heparin and assayed against the International Standard has been used.

For the experiments on the recovery of added heparin, Connaught heparin was used. The lot used was found to have 90 % of the metachromatic activity of the International Standard. The difference represents the water content of the sample.

RESULTS

In Table 1 is shown the recovery obtained by the method of commercial heparin added to canine blood. From 0·02 to 0·36 mg. of heparin was added to a total volume of 10 ml. The average recoveries in two series were 87 and 90 %. The recovery was as high with the lowest concentration of heparin (0·002 mg./ml.) as with the highest (0·036 mg./ml.). As shown on comparing series B with series A, incubation of heparin with blood at 37° C. for 3½ hr. had no effect on its recovery. Recovery of the heparin on addition to the plasma of the same blood was complete (98 %). This suggests that the 10 % not recovered on the addition of heparin to blood is due to loss of heparin in the cells. This is partly due to failure completely to remove the plasma from the centrifuged cells. It may also be partly due to adsorption of heparin on the white cells as reported by Fischer (1936). While routine recoveries are not always as high as in this series, recoveries are always better than 80 % of the added heparin, indicating that losses due to the manipulative procedures are less than 10%. On the basis of these results, a correction factor of 12% is added to the found value to give the amount of heparin present in samples of dog blood.

TABLE 1. Recovery of heparin after addition to dog blood

	Heparin recovered					
Heparin added (free acid, mg.*)	A		B		C	
	mg.	%	mg.	%	mg.	%
0·000	0·000	—	0·000	—	0·000	—
0·022	0·020	92	0·019	87	0·022	100
0·045	0·038	85	0·041	91	0·039	87
0·090	0·072	80	0·081	90	0·098	109
0·181	0·156	86	0·172	95	0·172	95
0·362	0·326	90	0·312	87	—	—
Average	—	87	—	90	—	98

A, 1·0 ml. of heparin solution added to 9·0 ml. of citrated blood. B, Same but blood plus heparin incubated for 3½ hr. before centrifuging. C, 1·0 ml. of heparin solution added to the plasma from 9·0 ml. of citrated blood.

* To convert to International anticoagulant units, for beef heparin multiply by 148. For other species of heparin, this factor must be determined (cf. Jaques & Charles, 1941).

TABLE 2. Recovery of heparin after addition to human blood

	Heparin recovered							
Heparin added (free acid, mg.)	Blood A				Blood B		Blood C	
	mg.	%	mg.*	%	mg.	%	mg.	%
0·000	0·000	—	0·000	—	0·005	—	0·000	—
0·022	0·011	50	0·011	50	—	—	0·015	68
0·045	0·029	65	0·030	67	0·027	60	0·025	56
0·045	0·030	67	0·028	62	—	—	0·033	73
0·090	0·068	76	—	—	—	—	0·055	61
0·181	0·123	68	—	—	0·167	92	0·117	65
0·362	0·292	81	—	—	0·296	82	0·232	64
Average	—	68	—	—	—	78	—	65

1 ml. of heparin solution added to 9·0 ml. of blood.

* From serum: for this the citrate was omitted, the blood allowed to clot, and the serum removed next morning.

Table 2 shows recoveries obtained with human blood. The average recovery was 18% lower than obtained with canine blood, i.e. ran from 60 to 80% of the amount of heparin added. This means that a larger correction factor (+31%) must be added to the values obtained for human blood. As shown in Table 2, no difference between plasma and serum has been found for the recovery of heparin.

As shown in Table 2, blood sample B, without addition of heparin, yielded a material by this method which showed metachromatic activity. The amount of this material in 10 c.c. of normal blood is usually below the sensitivity of the metachromatic test. Treatment of 40 ml. of human blood provided sufficient material for tests of metachromasia, in which the material showed qualitatively the same metachromatic powers as heparin. The material also showed good antithrombin activity. These results have indicated that the normal concentration of 'heparin' in human blood is of the order of 0·0001 mg./ml. Studies of this material are now in progress. Canine blood yielded a precipitate by this method but only occasionally did this material show metachromasia.

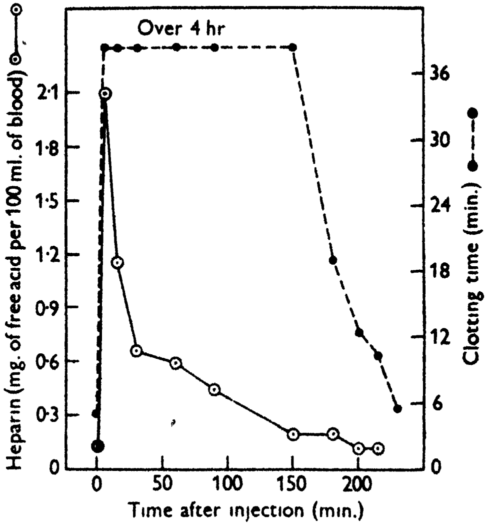

Fig. 2. Clotting times and blood-heparin concentrations after intravenous injection of heparin in the dog. Dog of 22·5 kg. given 45 mg. of sodium heparin at zero time. Concentration of heparin expressed in mg. of free acid per 100 ml. of blood.

In Fig. 2 is shown the clotting time and blood-heparin concentration for a dog weighing 22·5 kg., following the intravenous injection of 45 mg. of sodium heparin. Immediately after the injection the blood-heparin concentration was 0·021 mg./ml. expressed as free acid heparin. Calculated from the quantity of heparin injected and the blood volume (assumed to be one-eleventh of the body weight) the blood-heparin concentration should have been 0·0175 mg./ml. This indicates that the method is satisfactory for the determination of heparin added to blood *in vivo*. Also of interest as a new observation is the very rapid fall in the blood-heparin concentration until a level of 0·005 mg./ml. (expressed as free acid heparin) is reached. Thereafter the disappearance of heparin from the blood is more gradual. The clotting time only returned to measurable values 3 hr. after the injection, when the blood-heparin concentration was 0·0017 mg./ml. The clotting time then rapidly returned to normal with the further decrease in blood-heparin concentration.

SUMMARY

1. A method for the separation of heparin from blood is described.

2. Blood is collected in citrate and the heparin precipitated from the plasma with N-octylamine. The heparin is reprecipitated with brucine.

3. Recoveries of 80–90% are obtained for heparin added to blood, while complete recovery of added heparin is obtained from plasma.

This study has been greatly assisted by grants from the John and Mary R. Markle Foundation to the University of Toronto and the University of Saskatchewan. The senior author wishes to express his appreciation of the interest and support of Prof. C. H. Best, of the University of Toronto, in these studies and the assistance of Mrs Ursula Dale Irish, Miss Ruth Riggins, Mrs Ann Ricker and Mr E. Napke who took part in earlier investigations at the University of Toronto, which led to the successful development of the present method. Shawinigan Chemicals Ltd., Montreal, kindly supplied the octylamine.

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THE ROLE OF THE ADRENAL CORTEX IN THE MAINTENANCE OF THE SCIATIC PRESSOR REFLEX

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Cleghorn, Armstrong, Austen & McVicar (1941) were unable to confirm my findings that secretion from the adrenal cortex was an essential factor in the maintenance of post-ganglionic sympathetic neuromuscular conduction (Secker, 1938, 1939). They maintained their adrenalectomized cats by cortin administration, which was discontinued some 9–10 days before the central end of the splanchnic or sciatic nerve was stimulated. The animals, which were then showing signs of a mild or moderately severe adrenal insufficiency, gave a pressor response not significantly less than that given by cortin-treated controls provided the initial blood pressure had not fallen to shock level. The failure to obtain a pressor response to stimulation under conditions of low blood pressure they attribute to ganglionic failure. No attempt was made to test the effect of cortical extract after ganglionic failure. The failure to restore blood pressure in adrenalectomized dogs after traumatic shock had been shown by Swingle, Parkins, Taylor & Hays (1938), who further found that the administration of cortical hormone in adequate amounts restored the collapsed circulation to normal. Adrenalectomized cats, maintained on cortical extract for 4–26 days, gave normal carotid pressor reflexes but even 'in moderate states of insufficiency this reflex is practically abolished' (Nowak, 1938).

In experiments reported previously (Secker, 1938, 1948) it could be seen that the retraction of the nictitating membrane in response to stimulation of the cervical sympathetic trunk was accompanied after a short latent period by a pressor or depressor response, which, in adrenalectomized cats, gradually diminished as the retractor response failed and was restored by the administration of cortical extract. It was, therefore, thought worth while to stimulate the central end of the cut sciatic nerve in acutely adrenalectomized cats and, if the vasomotor response were reduced on repeated stimulation, to administer cortical extract and restimulate.

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* The paper has been prepared for publication by Prof. D. Burns.

METHOD

Cats were anaesthetized with chloralose (0·08 g./kg.) intravenously after induction with ether. In eight of the eighteen experiments Dial Liquid Compound 'Ciba' (0·08 g./kg. diallylbarbituric acid) was given intraperitoneally without ether induction. Both vagi were cut in the neck. Arterial blood pressure was recorded from the left common carotid artery and injections were made into the right femoral vein. The left sciatic nerve was cut and the central end enclosed in shielded electrodes or in a liquid electrode (Brown-Garry-Collinson type). Shocks were applied from an induction coil fed from a 4 V. accumulator. The stimuli were maximal and were applied for 30 sec. at 50/sec. The adrenals were excised after complete ligation, usually after a series of normal responses to stimulation had been obtained to determine the strength of stimulus to use. Once this had been determined, the electrodes were not disturbed during the course of the experiment. Most of the cats used were, as usual, females and their ovaries were removed as a routine precaution (Secker, 1948).

RESULTS

In two of the eight experiments in which dial was used, the response obtained, irrespective of the frequency or strength of the stimulus, was of the depressor type. In all the remaining experiments, six with dial and ten with chloralose, a pressor response was obtained.

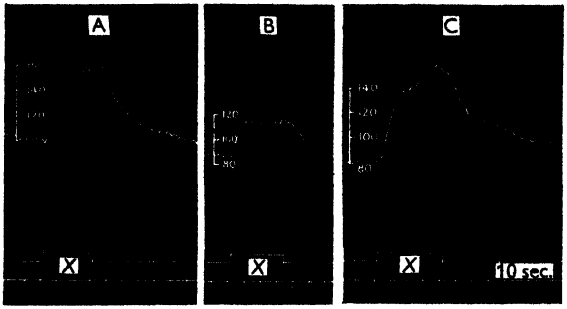

Fig. 1. Cat, ♀; 2·55 kg. Dial. Adrenals and ovaries removed. In each trace at *X* the sciatic was stimulated for 30 sec. at 50/sec. Coil at 8 cm. A, 25 min. after adrenalectomy. B, Between A and B, the sciatic was stimulated in 30 sec. bursts for 8 min. C, Between B and C, the sciatic was further stimulated in all for 32 min. and 12 min. before C, 1 ml./kg. eschatin was given. Time in 10 sec.

Removal of the adrenals followed by repeated stimulation of the central end of the cut sciatic nerve always resulted in a gradual diminution in the extent and duration of the response, whether pressor or depressor. In some experiments the stimulation was repeated till the response was extinguished.

In all but two of the experiments in which 1 or 2 ml. of eschatin (Parke Davis and Co.) was given after the response had been greatly decreased or eliminated after adrenalectomy, the response was fully or almost fully restored. In both of the failures, there was a markedly decreased response after adrenalectomy but no restoration by eschatin in one and only an insignificant response in the other. The extract used in these two experiments had been in the laboratory for some time.

Fig. 1 shows a typical trace. The pressor response to maximal sciatic stimulation is shown in A, 25 min. after adrenalectomy. In B, the pressor response to the same stimulus has decreased after sixteen periods of stimulation of 30 sec. each. Twelve minutes after 1 ml./kg. eschatin, given in the interval between B and C, the response has been restored.

Cleghorn *et al.* (1941) reported failure of the reflex pressor response only when the blood pressure had fallen to shock level. The present results are not in agreement with this observation. In Fig. 2, the level of blood pressure was artificially lowered between traces B (118 mm. Hg) and C (63 mm. Hg) and yet in trace D (65 mm. Hg) a pressor response is seen 10 min. after the injection of 1 ml./kg. eschatin.

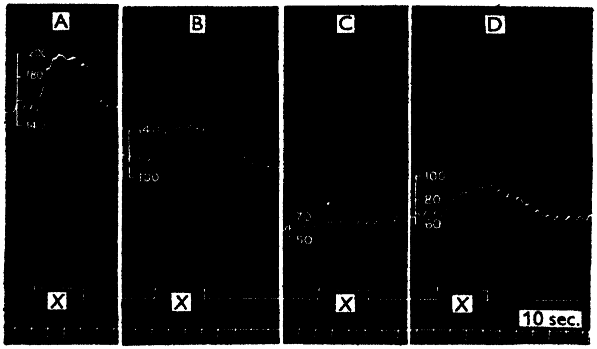

Fig. 2. Cat, ♀; 1·75 kg. Chloralose after ether induction. A, sciatic stimulation for 30 sec. at 50/sec. at *X*. Coil at 8 cm. Time in 10 sec. B, sciatic stimulation at *X*, 11 min. after adrenalectomy and ovariectomy (between A and B adrenals and ovaries removed). C, blood pressure lowered by eserine (0·3 mg./kg.) and sciatic stimulated 15 min. after B. During the interval the nerve had been stimulated for periods amounting to 2 min. D, sciatic stimulated 41 min. after adrenalectomy and 10 min. after eschatin (1 ml./kg.).

DISCUSSION

These experiments confirm the results of Swingle *et al.* (1938) and of Nowak (1938), but are not in agreement with those of Cleghorn *et al.* (1941). They support the hypothesis that an intact adrenal cortex is necessary for the efficient and prolonged action of the sympathetico-muscular system in cats. The experiments differ from those of Cleghorn *et al.* in three main respects, in that acutely adrenalectomized cats were used as against their cortin-maintained animals, in that the stimuli were maximal rather than supramaximal, and, most significantly, in that only male cats or ovariectomized females were used (Secker, 1948). The conclusion cannot be avoided that under the conditions of our experiments, reflex vasomotor responses gradually fail to occur after removal of the adrenals (and ovaries). These responses can be restored, irrespective of the level of blood pressure, by the injection of a cortical extract (or of progesterone). Therefore, some circulating sterol complex is necessary for the activity of sympathetic system. Previous experiments have indicated that the site of action of the cortical substance lies post-synaptically. The action may be either in the post-ganglionic neurones or in the neuromuscular (or neuroglandular) junctions.

SUMMARY

The vasomotor response to central sciatic stimulation in the cat steadily declines in intensity with repeated stimulation after acute adrenalectomy and may be extinguished.

Intravenous administration of an active extract of the adrenal cortex restores the response to normal or near normal.

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REACTIONS OF THE PERIPHERAL CIRCULATION TO EXTERNAL HEAT

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The decrease in blood flow to the skin that follows a fall in environmental temperature is generally considered to be due partly to reflex action arising from the stimulation of the cold receptors in the skin (Francois-Franck, 1876) and partly to a direct central effect of minute fluctuations in blood temperature (Pickering, 1932). The efferent vasoconstrictor pathway is the sympathetic supply to the skin (Freeman, 1935), and the vasoconstriction it mediates in response to cold may well be a protective temperature regulating mechanism (Deighton, 1933).

Barbour (1921) has pointed out that whereas a vasoconstrictor response to cold has obvious implications as regards temperature regulation, vasoconstriction in response to temperatures hotter than the body might be equally effective in protecting the body against heat. Martin & Jacoby (1922), Pickering (1932), and other workers, have indeed observed that heat stimulus can produce vasoconstriction, though quantitative studies of the effect have not been made.

In the present paper a further study of the effects of external heat on the peripheral circulation is described. Finger blood flows, forearm blood flows and skin temperatures have been measured on healthy human subjects under varying conditions of environmental temperature.

METHODS

Blood flows were measured in human subjects using venous occlusion plethysmographs according to the principles of Hewlett & Van Zwaluwenberg (1909). Finger blood flows and forearm blood flows were estimated separately to distinguish between the vascular reactions of skin and muscle in the manner suggested by Grant & Pearson (1938).

Measurement of finger blood flows. The plethysmograph was based on a design suggested by A. A. Harper (Fig. 1). It consisted of a metal canister which contained four fingers of the hand. In one series of experiments it was air-filled and was sealed to the base of the digits by means of vaseline bandages, the flange (Fig. 1) being omitted. In more recent experiments the canister was sealed to the hand at the level of the metacarpal heads by means of a diaphragm about ¼ in. thick, made from expanded rubber fixed in position on the plethysmograph between the flange and the plate. (Rubazote floor covering made by the Expanded Rubber Company, Croydon, is suitable. It has hard, non-porous surfaces which can be stripped off leaving soft spongy rubber suitable for use in the plethysmograph.) The diaphragm had a central aperture just large enough to fit the hand, the soft expanded rubber automatically effecting a simple airtight seal with negligible pressure. The plethysmograph was immersed in a water-bath thermostatically maintained at 31° C., water circulating continuously from the bath through the instrument between readings. Two thermocouples were incorporated, one to check the water temperature inside the apparatus and the other to register skin temperatures from the middle finger. The advantage of this type of plethysmograph was that although the muscular elements of the hand were excluded, much larger volume changes could be recorded than with a plethysmograph enclosing only one digit. In addition, local temperatures could be accurately measured and controlled. Readings were taken only when the water temperature was within 0·05 of 31° C.

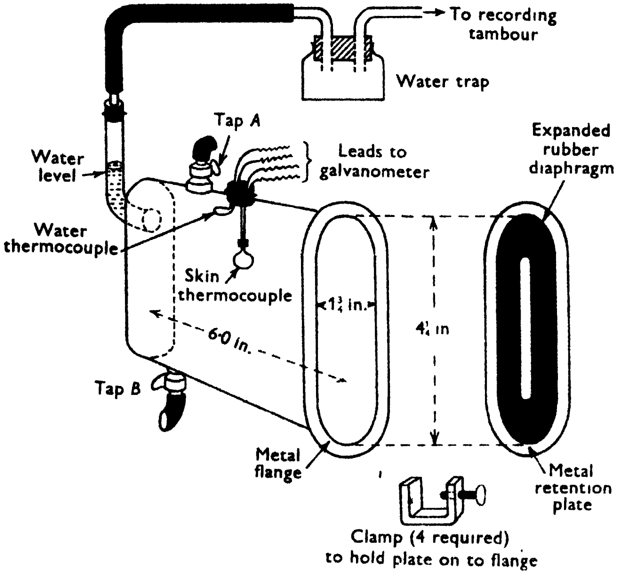


Fig. 1. Finger plethysmograph. Taps *A* and *B* used for continuous circulation of water between readings.

The collecting cuff (venous tourniquet) was at the wrist; optical recording was employed, using a Wigger's capsule and a recording camera.

Forearm blood flows. For these measurements a plethysmograph similar to that described by Grant & Pearson (1938) was used. The ends were closed by diaphragms made from 'Rubazote', sealed to the arms by means of thin rubber sleeves. When finger and forearm blood flows were measured simultaneously on the same arm, the forearm and hand were immersed in a large water-bath thermostatically maintained at 31° C. The finger blood flow was recorded first, using a cuff pressure of about 55 mm. of mercury; pressure in the wrist cuff was then raised to occlude the hand circulation and the forearm blood flow taken with a cuff pressure of 60 mm. Hg on the upper arm.

A kymograph and float volume recorder was generally used in the estimation of forearm blood flows, although in some cases the optical apparatus was employed. For the purpose of the present investigation the difference in accuracy was not found to be significant.

Skin temperature measurements. Skin temperatures were measured thermoelectrically using skin thermocouples incorporated in the plethysmograph, and a moving coil mirror galvanometer calibrated to read directly changes of 0·02° C. The thermocouples were made from 40 s.w.g. copper and constantan wire and applied to the finger in the form of a loop, the size of which could be varied by means of a small rubber 'slider'. Cold junction temperatures were measured with a standard thermometer reading to 0·1° C. (N.P.L. certificate of accuracy).

The heating cabinet. An electrically heated cabinet was used to control the environmental temperature. The subject sat fully enclosed within the cabinet except that one arm projected into a water-bath through an aperture in the cabinet wall. The air inside the cabinet was distributed by means of a small fan, a series of baffle plates preventing draughts from impinging on the subject. The temperature distribution inside remained uniform over the whole range of heating. Thermostatic control enabled the cabinet to be maintained at any desired temperature up to 50° C.

The subjects. The subjects were healthy male students, naked or wearing swimming trunks. They remained seated inside the cabinet throughout the experiment, which was conducted, without talking, in a quiet room.

RESULTS

The effect of rising environmental temperatures

A series of experiments was performed in which the subject was exposed within the cabinet to a rising environmental temperature. Records were made of blood flow in the fingers and forearm, rectal or mouth temperatures, skin temperatures from the fingers, and, in two experiments, of the onset of perspiration.

Blood flow responses in the fingers. In the nine cases in which the water-filled plethysmograph was used, similar results were obtained (Table 1). There was a rise in skin blood flow during the early stages of heating, followed by a fall, followed in turn by a final rise:

(*a*) As the cabinet temperatures rose from 25° to about 36° C. the blood flow in the fingers increased gradually from about 4 c.c./100 c.c./min. to about 10 c.c./100 c.c./min.

(*b*) As the cabinet temperature rose from about 36° to about 40° C. there was a fall in blood flow. Between 36 and 38° C. the mean fall in blood flow was 3·02 c.c./100 c.c./min. The standard error was 0·616. The figures recorded in Table 1 are thus significant.

(*c*) As the cabinet temperature rose above 40° C. a final increase occurred in skin blood flow which rose up to or beyond the previous maximum.

A typical experimental result is shown graphically in Fig. 2.

In an earlier series of seventeen experiments, using an air-filled plethysmograph, similar results were obtained. In each case, after a preliminary rise in blood flow, there was a fall, beginning at an average cabinet temperature of 36·5° C., followed by a rise in blood flow beginning at an average cabinet temperature of 39·9° C.

Skin temperature responses in the fingers. The changes in skin temperature as

Table 1. Finger blood flow changes during rising environmental temperatures (c.c./100 c.c./min.)

Exp. no.	Cabinet temperatures (° C.) 25	30	36	38	41	45
33	5·9	7·0	10·1	7·5	9·0	—
26	7·2	10·4	12·2	7·0	8·0	11·6
30	3·1	3·5	7·5	6·7	5·5	7·0
41	—	5·2	9·0	6·6	8·1	9·5
43	4·4	5·9	7·7	5·3	6·2	10·0
46	4·6	6·0	7·1	4·9	6·1	7·7
49	—	4·0	7·4	5·9	—	—
50	4·0	5·6	10·0	6·6	4·5	12·5
70	4·6	4·8	10·9	4·2	8·8	10·9

Water-filled plethysmograph used in all cases at 31·0° C. (±0·03° C.). Experiment numbers included for comparison with Tables 2, 3 and 4.

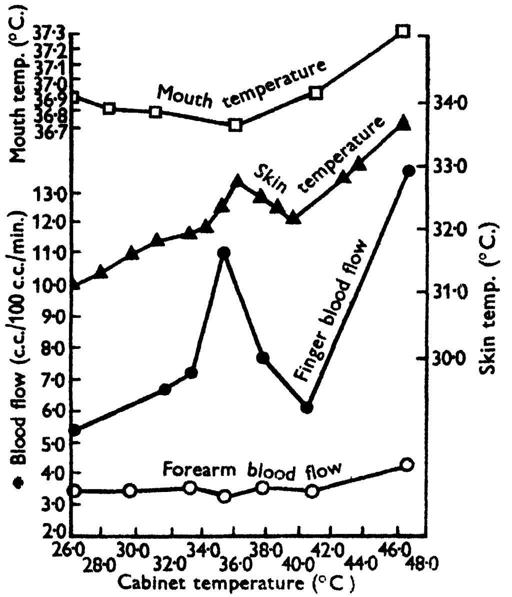


Fig. 2. The effects of an evenly rising cabinet temperature on the human subject. Simultaneous records of forearm and finger blood flows, skin temperatures from right middle finger and mouth temperatures.

the environmental temperature increased were similar to the changes in finger blood flow (Table 2):

(*a*) There was an increase in skin temperature as the cabinet temperature rose from 25° to about 36° C.

(*b*) Between cabinet temperatures of 36° and about 40° C. there was a drop in skin temperature. Analysis of the figures shows a mean drop of 0·42° C. between cabinet temperatures of 36 and 38° C., with a standard error of 0·09. The fall in skin temperature is thus statistically significant.

(*c*) There was a final increase in skin temperature when the cabinet temperature rose above 40·0° C.

TABLE 2. Skin temperature changes (° C.) of middle finger during rising environmental temperatures

Exp.	Cabinet temperatures (° C.)						
no.	25	30	36	38	41	45	50
41	—	31·3	33·5	32·9	33·2	33·6	—
43	31·0	31·3	32·2	32·4	32·6	32·7	—
33	31·0	31·3	31·7	31·1	31·4	—	—
32	32·1	32·6	32·9	32·5	32·0	32·5	32·8
30	31·5	32·5	34·7	34·4	35·1	35·1	—
46	31·8	32·0	32·5	31·8	32·2	32·4	—
56	31·0	32·0	33·1	32·7	32·5	33·7	—
70	31·3	31·6	32·3	31·9	31·2	32·0	—
71	31·8	32·5	32·8	32·2	32·9	33·3	—

All records made inside plethysmograph in water-bath at 31° C. (±0·03° C.). Experiment numbers included for comparison with Tables 1, 3 and 4.

Blood flow responses in the forearm. The average resting blood flow in the forearm was found to be 2·1 c.c./100 c.c./min. It was not affected by changes in external temperature during the earlier stages of heating. A rise in forearm flow began at an average cabinet temperature of 42° C. The average maximum blood flow produced in the forearm by heating up to 50° C. was 10·0 c.c./100 c.c./min.

A typical response is seen in Fig. 2. Table 3 gives the results of three cases in which optical recording was used.

TABLE 3. Forearm blood flow changes (c.c./min.) during rising environmental temperatures

Exp.	Cabinet temperatures (° C.)					
no.	23	30	36	38	41	45
49	2·5	2·5	2·4	2·3	2·4	7·3
50	2·2	2·5	2·5	2·3	4·5	7·2
66	2·0	2·2	2·1	3·4	4·7	8·5

Water-filled plethysmograph at 31° C. with optical recording. Experiment numbers included for comparison with Tables 1, 2 and 4.

A further six cases where kymograph recording was used gave similar results.

Body temperature variations during external heating. The results obtained in eight heating experiments when the rectal temperature was recorded thermoelectrically are given in Table 4. Results have been calculated to the nearest 0·1° C.

In all other experiments mouth temperatures were recorded either thermoelectrically or by means of a clinical thermometer. The results were similar to the rectal temperature records. During the early stages of heating, between 25 and 36° C., the body temperature remained steady or fell slightly. In most cases it began to rise at a cabinet temperature of 38–40° C., the maximum rise occurring at cabinet temperatures above 40° C.

Table 4. Rectal temperature changes (° C.) during rising environmental temperatures

Exp. no.	Cabinet temperatures (° C.) 25	30	36	38	41	45
33	—	—	35·7	35·7	35·9	—
30	—	36·9	36·9	36·9	37·0	—
41	37·1	37·1	37·2	37·3	37·4	—
43	37·0	37·0	37·0	—	37·4	37·6
46	37·0	36·8	36·9	37·0	37·3	37·6
49	36·9	36·8	—	36·7	37·0	37·4
50	36·9	36·8	36·7	36·8	36·9	37·3
70	38·0	—	37·7	37·6	38·0	38·5

All temperatures recorded thermoelectrically to the nearest 0·1° C. Experiment numbers included for comparison with Tables 1, 2 and 3.

The onset of perspiration. Two experiments were performed using a starch/iodine indicator applied to the forehead, chest, abdomen, thighs, feet and the forearm of the subject. Both experiments produced similar findings; the results of one experiment are shown in Fig. 3.

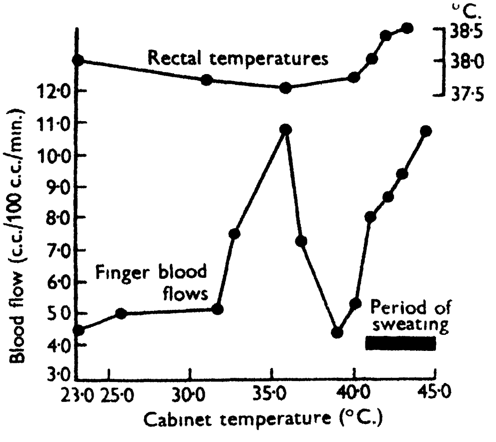


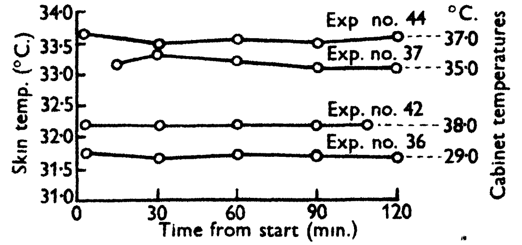
Fig. 3. The onset of perspiration during external heating.

Perspiration began after the body temperature had risen, when the cabinet temperature had reached 40° C. In both cases a significant vasoconstriction occurred before the onset of perspiration, while the cabinet temperature was rising from 36 to 40° C.

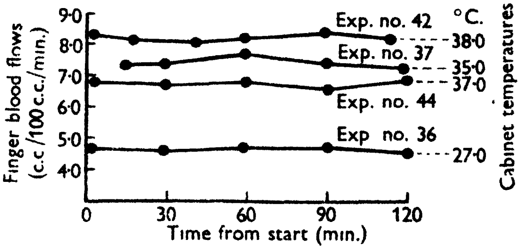
The effect of constant environmental temperatures on blood flow.

A number of experiments were performed in which the cabinet was kept at a constant temperature for periods up to 2 hr. In the experiments recorded in Figs. 4 and 5 no significant variation in blood flow took place.

The vasoconstriction recorded previously at cabinet temperatures higher than 36° C. was not therefore due to fatigue of the subject and bore no relation to the duration of the experiment.



A. Skin temperature responses



B. Finger blood-flow responses

Fig. 4. Control experiments. simultaneous records of skin temperatures and finger blood flows at various environmental temperatures.

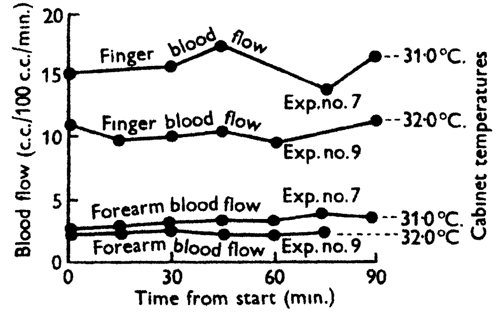


Fig. 5. Control experiments: simultaneous records of finger and forearm blood flows at different environmental temperatures.

The effect of falling environmental temperatures

A number of experiments were performed to investigate the blood flow responses during the cooling of a subject who had already been heated.

Blood flow responses to cooling before the rise in body temperature. In four experiments the cabinet temperature was raised to 40° C. then allowed to fall. The heating and cooling each took about an hour; rectal temperatures were recorded and showed no rise. Fig. 6 shows a typical result.

In each case the finger blood flow increased as the cabinet temperature rose to about 36° C., then dropped significantly as the cabinet temperature rose to 40° C. As the cabinet temperature fell to about 36° C. the blood flow rose again to about its previous maximum, then fell to its initial starting level as the cabinet cooled to room temperature.

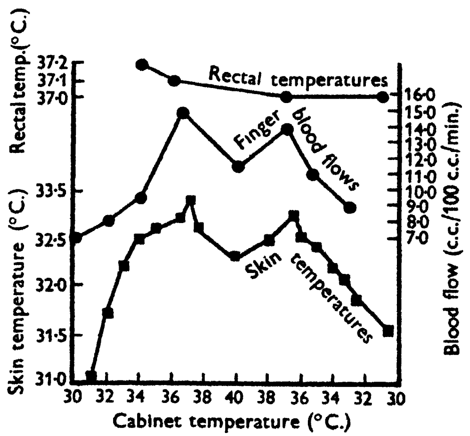


Fig. 6. Reversibility of blood flow responses to environmental temperature changes, before the rise in body temperature.

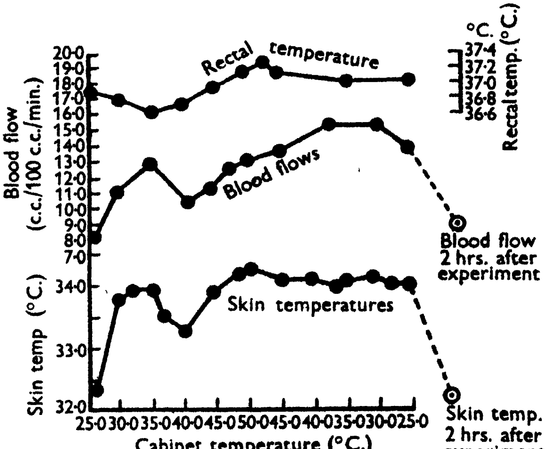


Fig. 7. Finger blood flow responses to lowering the environmental temperature after the rise in body temperature.

Blood flow responses to cooling after the rise in body temperature. In three experiments the cabinet temperature was raised to 50° C. then allowed to cool again. Finger blood flow records from one experiment and skin temperature records from another are shown in Fig. 7. Normal curves were obtained in each case as the cabinet temperature rose and in all three experiments a rise in rectal temperature of about 1° C. was recorded during the last phase of heating.

When the cabinet was allowed to cool, however, the blood flow remained elevated until the end of the experiment. The heating curve was not retraced and it was not until 2 hr. later, when the body temperature had returned to normal, that the finger blood flow fell to its starting level.

The effect of sympathectomy on blood flow responses to changes in environmental temperature

Fig. 8 shows the effect of preganglionic cervical sympathectomy on a patient suffering from causalgic pain in the fingers who gave a normal triphasic response to rising environmental temperatures before operation. The heating tests were repeated 3 weeks after operation when it was found that all sensitivity to environmental temperature changes had been lost, though the postoperative level of blood flow was considerably lower than the preoperative maximum.

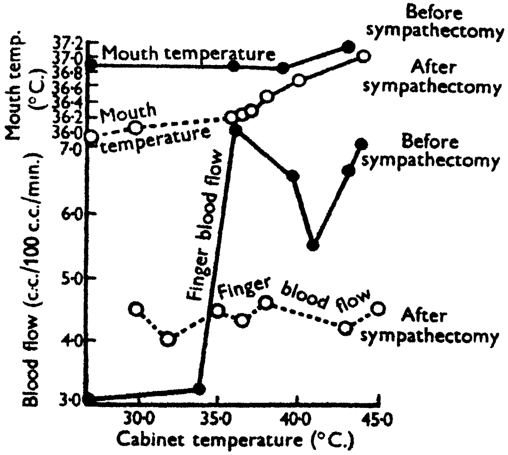


Fig. 8. The effect of sympathectomy on blood flow responses to rising environmental temperatures.

Records of forearm flow responses from other cases before and after preganglionic section showed also that the increase in muscle flow produced in these experiments by body heating did not occur after section of the sympathetic nerves.

DISCUSSION

During exposure of the human subject to rising environmental temperatures, three distinct phases could be distinguished in the vascular responses of the skin and the forearm muscle:

(*a*) An initial phase of cutaneous vasodilatation as the environmental temperature rose to about 36° C., with no change in muscle blood flow.

(*b*) A phase of cutaneous vasoconstriction as the temperature rose from 36 to about 40° C., again with no change in muscle blood flow.

(*c*) A final phase of vasodilatation in both skin and muscle as the temperature rose above 40° C.

The nature of the response. During phases (*a*) and (*b*) the rectal temperature remained steady or fell slightly; there was no sensible perspiration and the changes in the skin circulation were reversible by lowering the environmental temperature, always provided that the body temperature had not risen. During phase (*c*), however, the rectal temperature rose, perspiration began and the increase in skin blood flow was maintained irrespective of the environmental temperature, until the body temperature returned to normal. Control experiments performed at a number of different environmental temperatures, demonstrated that the responses of the skin circulation during the first two phases, (*a*) and (*b*), bore no relation to the length of time the subject was maintained at any one temperature, as long as the body temperature did not rise.

The evidence suggests that whereas exposure of the body to environmental temperatures below 36° C. leads to a vasoconstriction, exposure of the body to temperatures above 36° C. also leads to a vasoconstriction. During an experiment in which the environmental temperature is raised it is reasonable to suppose therefore that the initial vasodilatation of phase (*a*) is due to the removal of the vasoconstrictor response to a cool environment. The vasoconstriction of phase (*b*) is a positive response to environmental temperatures higher than 36° C. The final dilatation of phase (*c*) is probably caused by the rise in body temperature.

Experiments performed on patients before and after sympathectomy indicate that the efferent pathway for all these effects is in the sympathetic nerves. Cutaneous vasoconstriction in response to cold has been regarded as part of the temperature regulating mechanism. It is possible that the vasoconstriction described in response to temperatures higher than the body has equal value in protecting the body against heat.

SUMMARY

1. An improved plethysmograph is described for the measurement of finger blood flows, and a technique is described for the estimation of blood flow changes in the fingers and forearm in response to changing environmental temperatures.

2. The response of the skin circulation to rising environmental temperatures is described. It consists of:

(*a*) An increase in skin blood flow as the environment warms to approximately 36° C.

(*b*) A decrease in skin blood flow as the environment rises in temperature from 36 to 40° C.

(*c*) An increase in blood flow as the environmental temperature rises above 40° C.

During phase (*a*) and (*b*) the body temperature remains steady or falls, and there is no change in muscle blood flow.

During the final phase (*c*) the body temperature increases, forearm blood flow rises and perspiration begins.

3. The vasoconstriction that occurs in the skin when the environmental temperature rises above 36·0° C. may be a temperature regulating mechanism.
4. Forearm blood flows do not respond to environmental temperature changes until the body temperature has risen.
5. The efferent pathway for these vascular effects is in the sympathetic nerves.

The author wishes to express his thanks to Dr A. A. Harper and to Prof. F. H. Bentley for constant help and advice throughout the work.

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EFFECT OF VOLUNTARY HYPERPNOEA ON PULMONARY BLOOD FLOW

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During the course of certain experiments on the mechanism of pulmonary ventilation during large breaths it was noted that the amount of oxygen absorbed was unexpectedly large. It was decided to investigate this phenomenon in detail.

METHODS

Determinations, as far as possible, were done in the early afternoon. The subject (G.H.A.) rested in the position in which the subsequent experiment was to be performed (i.e. either sitting upright or lying flat) for 10 min. The rate of oxygen uptake was then determined by spirometer or Douglas bag; and 5 min. later the experiment was performed. (In that interval, especial care was taken that the subject should refrain from activity calculated to alter the respiratory rhythm.)

An experiment involved the collection of 1–5 vital capacity breaths in a Douglas bag, previously rinsed with expired air. An end expiration sample of the last breath prior to collection was taken and a similar final sample taken at the end of the last collected expiration. The time between the specimens was recorded. A 10 l. Douglas bag was used where possible, otherwise one of standard size. The bag was fitted with a two-way tap—opened and closed by the subject between breaths—connected to a short wide mouthpiece. Although the manipulation of the tap between each breath involved the possibility of a slight loss of expired air, this was preferred to the risk of altering the results by using a valve with its inevitable slight resistance. The bag was sampled and emptied through a wet meter. Allowance has to be made for the higher oxygen contact of the alveolar air at the end of the large breaths; this accounts for a substantial proportion of the oxygen uptake during the time of the experiment. An example of the calculation is as follows:

1400 hr. 5 May 1948. Bar = 749 mm. $T = 21{\cdot}1^\circ$ C.
Subject G.H.A. Position: seated. Residual air vol. = 1·38 l. at N.T.P.
Metabolic rate determined before deep breathing = 320 c.c./min. at N.T.P.
No. of breaths = 2 in 14·6 sec.
Vol. collected in Douglas bag = 7·30 l. at N.T.P.

Analysis of specimens (mean of duplicates)

	O_2 (%)	CO_2 (%)	N_2 (%)	R.Q.
Initial alveolar sample	15·05	5·45	79·50	0·904
Collected expired air	18·40	3·59	78·01	1·576
Terminal alveolar sample	17·58	4·44	77·98	1·433

$$\text{Vol. of oxygen inspired} = \frac{7300 \times 20{\cdot}93 \times 78{\cdot}01}{100 \times 79{\cdot}04} = 1508 \text{ c.c.} \qquad (1)$$

$$\text{Vol. of oxygen expired} = \frac{7300 \times 18{\cdot}40}{100} = \underline{1343} \text{ c.c.} \qquad (2)$$

$$\therefore \text{Vol. of oxygen retained} = (1) - (2) = 165 \text{ c.c.} \qquad (3)$$

$$\text{Vol. of oxygen in residual air at end of deep breathing} = \frac{1380 \times 17{\cdot}58}{100} = 243 \text{ c.c.} \qquad (4)$$

$$\text{Vol. of oxygen in residual air at beginning of deep breathing} = \frac{1380 \times 15{\cdot}05}{100} = \underline{208} \text{ c.c.} \qquad (5)$$

$$\therefore \text{Vol. of oxygen retained but not absorbed} = (4) - (5) = 35 \text{ c.c.} \qquad (6)$$

$$\therefore \text{Vol. of oxygen absorbed} = (3) - (6) = 130 \text{ c.c.} \qquad (7)$$

But vol. of oxygen that should have been used in 14·6 sec. without hyperpnoea

$$= \frac{320 \times 14{\cdot}6}{60} = \underline{78} \text{ c.c.} \qquad (8)$$

$$\therefore \text{Excess oxygen uptake attendant upon hyperpnoea} = (7) - (8) = 52 \text{ c.c.}$$

A major possibility of error arises if either the last uncollected or the last collected expiration is incomplete. In the former event the effect would be to give an absorption in excess of real uptake for the measured period, in the latter case, to give a value erroneously low. With trained subjects neither error is likely to be substantial.

A second source of error arises if the alveolar sample does not truly represent the residual air composition at that moment. This error might be expected to be serious in emphysematous subjects.

A series of observations of oxygen absorption were made during breath-holding at full inspiration commencing and ending with full expiration. It is considered that up to the point at which alveolar oxygen content falls too low to secure 95% saturation this procedure is equivalent to a single large breath.

RESULTS

The amount of oxygen which would have been consumed in the same time during normal breathing was deducted from that observed during the periods of maximum breathing. The values obtained are given in Table 1 and Figs. 1 and 2.

TABLE 1

$a = 1$ max. breath; $b = 2$ max. breaths and $c = 3$–5 max. breaths

		Sitting	Lying
M.R. (c.c./min.)		313 ± 2·8 (26)	295 ± 6·4 (14)
Additional O_2 uptake (c.c.)	*a*	37 ± 3·9 (9)	12 ± 3·5 (5)
	b	48 ± 2·8 (6)	29 ± 5·0 (5)
	c	85 ± 8·0 (10)	32 ± 3·8 (5)
a : *b*	*t*	2·08	2·66
	P	∠0·1	∠0·05
b : *c*	*t*	4·30	0·36
	P	∠0·001	∠0·8

The additional oxygen uptake is much less in the horizontal position and shows no significant increase after 2 breaths, although in the sitting position there is a large increase in uptake in the 3–5 breath group, observations in which lie at the upper limit of the permissible duration, indeed, rather beyond this limit.

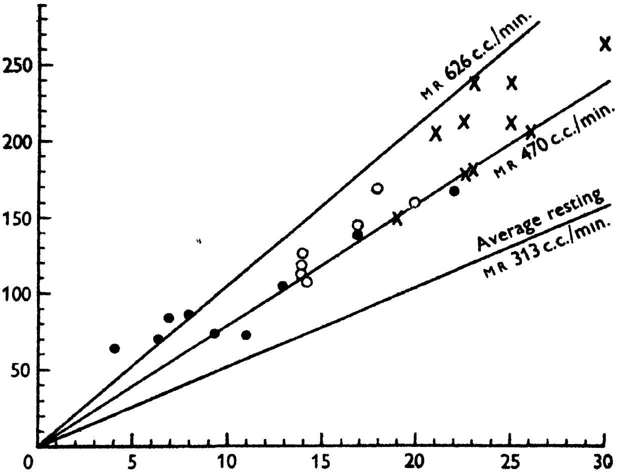

Fig. 1. Oxygen uptakes from 1–5 maximum breaths in sitting subject. ●, 1 breath; ○, 2 breaths; ×, 3–5 breaths. Lowest line: average resting M.R. (313 c.c./min. at N.T.P.). Middle line: resting M.R. + 50%. Upper line: resting M.R. + 100%. Abscissa: time in sec. Ordinate: O_2 uptake in c.c. at N.T.P. Subject: G.H.A.

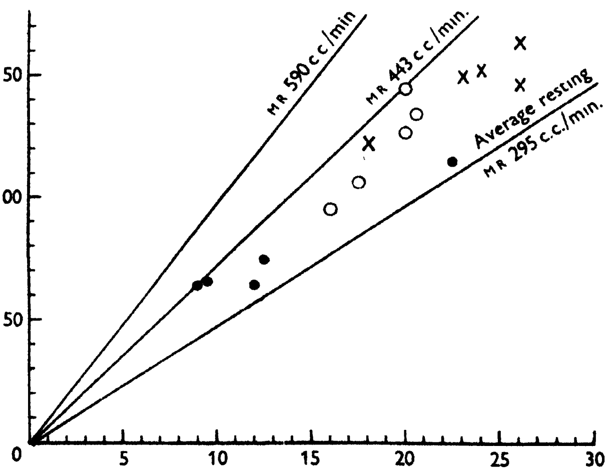

Fig. 2. Oxygen uptakes from 1–4 maximum breaths in recumbent subject. ●, 1 breath; ○, 2 breaths; ×, 3–4 breaths. Lowest line: average resting M.R. (295 c.c./min. at N.T.P.). Middle line: resting M.R. + 50%. Upper line: resting M.R. + 100%. Abscissa: time in sec. Ordinate: O_2 uptake in c.c. at N.T.P. Subject: G.H.A.

Fig. 3 shows the rates of oxygen uptake during the holding for various periods of breaths of maximum volume. Under 20 sec. the rates are far above the normal M.R., thus confirming the single maximum breath experiments.

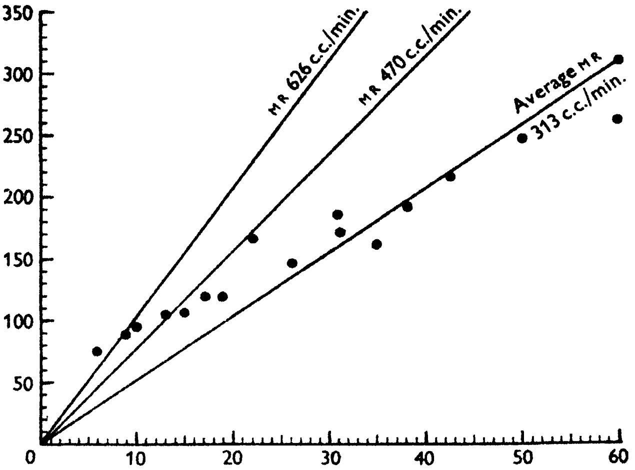

Fig. 3. Oxygen uptakes during breath-holding at full inspiration. (Time record is from end of expiration to end of expiration.) Points represent individual observations. M.R. lines as in Fig. 1. Abscissa: time in sec. Ordinate: O_2 uptake in c.c. at N.T.P. Subject: G.H.A.

DISCUSSION

It must be emphasized that our observations are limited to periods of deep breathing of less than 30 sec., mostly less than 20 sec. It is reasonable to assume that in such short periods the oxygen content of the blood entering the lungs does not greatly change, although from 15 sec. onwards increasing recirculation can be expected; the increasing scatter of the longer-period observations is probably explicable on this basis. Furthermore, the muscular effort of deep breathing leads to an increase in metabolic rate which can be expected to become apparent as soon as recirculation is substantial.

Krogh & Lindhard (1914) used fractional sampling methods to investigate gaseous exchanges during the respiratory cycle, and found that in a resting subject (J.L.) the oxygen absorption was high during inspiration and low during expiration, and that a voluntary large respiration at rest in the sitting position resulted in an increased oxygen uptake; they attributed this to increased flow of blood into the lungs. During the course of an investigation into the circulation time with reference to the acetylene method of determining cardiac output, Gladstone & Dack (1935) found that during the first 8 sec. of forced rebreathing the oxygen absorption was approximately twice the rate observed during the quiet breathing of the preceding metabolism test. A demonstration of this enhanced oxygen absorption was provided (Gladstone, 1935) by showing the alteration in a spirometer tracing of the resting respiratory level following a brief period of voluntary hyperpnoea. We have frequently repeated this demonstration, but have found the method unreliable for accurate quantitative work because of the tendency in many subjects of the resting respiratory level to become unstable following hyperpnoea.

The only conceivable explanation of the observed additional oxygen absorption is the entry into the pulmonary capillaries of blood additional to the resting flow. According to McMichael & Sharpey-Schafer (1944) the mean arterio-venous oxygen difference in the upright position is 6·1 vol. % so that an additional oxygen uptake of 80 c.c. implies the entry into gaseous equilibrium with alveolar air of an extra amount of blood of the order of 1·25 l., a reasonable quantity in an organ with such an accommodating capillary bed as the lung.

A major cause of this augmentation of flow into the pulmonary circulation is presumably the abnormal depression of intra-thoracic pressure during a large inspiration. Daly (1927) enclosed the heart and lungs of a heart-lung preparation in a box to which suction could be applied and found that deep respiratory movements could double the circulation. Wiggers (1935) states that 'the bulk of experimental evidence still supports the view that pulmonary peripheral resistance decreases during lung inflation; this decrease is due to the radial traction exerted upon pulmonary capillaries by the expanding alveoli'. Macklin (1946) made radio-opaque injection studies of freshly excised mammalian lungs at various stages of inflation. He concluded that the volume of the arteries and veins is increased in inflation as the result of their lengthening with retention of width or even widening produced by the pull of connective tissue attached to the expanding air sacs which surround the vessels.

It is known that inspiration is associated with a peripheral vasoconstriction (Stürup, Bolton, Williams & Carmichael, 1935), as demonstrated by a finger plethysmograph, and the existence of such a reflex has an attractive teleological explanation when it is known that a single deep breath is capable of attracting 10% of the total blood volume into the pulmonary circulation.

As regards the much smaller augmentation of oxygen uptake when deep breathing is performed in the horizontal position, a reduction of one-third was expected as McMichael & Sharpey-Schafer (1944) had shown that in the supine position the A–V O_2 difference was only 4 vol. %; however, the reduction observed was of the order of two-thirds suggesting the operation of some additional factor. McMichael & McGibbon (1939) studied the reduction in lung volume which occurs on assuming the horizontal position; they found no change in the size of the thoracic cavity and they concluded that the reduction in lung volume was due to increased pulmonary engorgement associated with the 25–30% higher cardiac output in this position. This factor probably accounts for a substantial part of the reduction in the excess oxygen uptake deep breathing, as the pulmonary vascular bed has already had part of its reserve capacity occupied. The fact that no further augmentation of oxygen uptake occurred after 2 breaths in the supine position suggests such a constricted range of expansion of the vascular bed as compared with the upright position.

It is reasonable to predict that an inverse relationship exists between the level of cardiac output and capacity to augment oxygen uptake by deep breathing as all the available evidence goes to show that, up to the limit of physiological adaptation, increased pulmonary blood flow is accommodated not by elevation of pulmonary arterial pressure with increased velocity but by enlargement of the capillary bed with an increase in instantaneous pulmonary blood volume. To test this hypothesis studies are being made in pathological states of high cardiac output—particularly these which are reversible—as in severe anaemia, hyperthyroidism, patent ductus arteriosus and accessible arterio-venous fistula.

SUMMARY

1. An attempt to use the concentration of alveolar oxygen to shed light on intrapulmonary gaseous distribution in deep breathing revealed an unexpectedly large oxygen absorption in voluntary hyperpnoea.

2. A more detailed study of this phenomenon showed that 1–5 maximal breaths in the sitting position led to an oxygen absorption of from 37 to 85 c.c. in excess of the amount predicted on the basis of the known metabolic rate. This implies the entry into the pulmonary circulation of an extra 1·25 l. of blood.

3. The same experiments performed in the horizontal position show the augmentation of O_2 uptake to be approximately one-third of that in the upright position. This is due to the smaller A-V O_2 difference and increased pulmonary blood volume in the supine position.

We are indebted to Messrs Imperial Chemical Industries Ltd. for a grant towards expenses, to assistance from Dr A. G. W. Whitfield, himself engaged on cognate problems, and to the industry and enthusiasm of our senior technician, A. C. Pincock.

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AIR DISTRIBUTION IN LUNGS DURING HYPERVENTILATION

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During research into some features of the response of the pulmonary circulation to high cardiac output, curiosity arose as to the mechanism of the adaptive expansion of the pulmonary vascular bed during the high cardiac outputs of exercise, severe anaemia, etc. It seemed possible that this adaptation might be a simple consequence of the alveolar expansion brought about by hyperpnoea. There was uncertainty whether deep breaths are accommodated by: (1) a further distension proportionate to their ventilation during quiet breathing, of those alveoli used at rest; (2) the opening up of successive ranges of 'reserve alveoli', which are folded up parachute-wise during quiet breathing; or (3) a mixture of these two mechanisms. If the first hypothesis be correct and alveolar ventilation uniform, it should be possible to predict, after any depth of inspiration, the final alveolar concentration of an inert 'reference gas' by the following formula

$$P_3=\frac{P_1V_1+P_2V_2}{V_1+V_2},$$

where P_1 = concentration of gas in alveolar air before inspiration;
P_2 = concentration of gas in inspired air;
P_3 = concentration of gas in alveolar air after inspiration;
V_1 = volume of air in lungs before inspiration;
V_2 = volume of inspiration.

(A correction must be added to the formula if the reference gas takes part during the period of the breath in any significant exchange with the blood).

The sample taken at the end of a complete expiration (hereafter called an end-expiration sample) is probably the most representative specimen of alveolar air that can be obtained as it is least likely to be influenced by contamination with dead-space air.

Any systematic irregularity of alveolar ventilation would cause a discrepancy between the alveolar concentration thus predicted and that observed by analysis of an end-expiration alveolar sample.

But, if the second hypothesis be correct, then, as the depth of inspiration increases, a rising proportion of the air inspired must enter newly opened alveoli and thus neither mix with the contents of the normally open alveoli, nor contribute to the subsequent end-expiration sample on the reasonable assumption that the air expired last originates in alveoli which are always 'open'. The latter will therefore exhibit, with deeper breaths, a progressive deviation from the composition predicted on the basis of the first hypothesis.

METHODS

Certain initial experiments were made using oxygen as reference gas, with a correction for uptake on the basis of a previous M.R. determination. The control experiments, with inspiration from the residual to the resting respiratory level, gave satisfactory results; but with inspiration to maximum capacity discrepancies were encountered to the significance of which consideration is given in a separate paper (Armitage & Arnott, 1949); these clearly indicated the unsuitability of oxygen for this purpose. Instead hydrogen, helium and nitrogen were employed as reference gases; none of which involved any significant correction for transfer across the alveolar membrane within the time intervals concerned.

Four healthy males, aged 39, 30, 20 and 22, all accustomed to the experimental procedures, served as subjects. The subdivisions of lung volume were measured using a Benedict-Knipping spirometer with a Shakspear katharometer, employing the constant-volume hydrogen (or helium) dilution method (McMichael, 1939).

Experiments using hydrogen

Inspiration of air into an air-hydrogen alveolar mixture. With the subject seated in circuit with the Benedict-Knipping spirometer (circuit *A*), an air-hydrogen mixture was breathed as in the lung-volume estimation procedure. When the katharometer reading indicated the attainment of hydrogen-equilibrium in the system, the subject detached himself at the end of a normal expiration and, turning his head to an adjacent mouthpiece leading from an air-filled Benedict-Roth spirometer (circuit *B*), took a measured inspiration of air. Thereafter, an end-expiration alveolar sample was taken in the usual way in a sampling-tube of 100 c.c. capacity. The sample was then passed through the katharometer and its hydrogen content determined with appropriate corrections for the CO_2 content. This was compared with the value predicted by the formula on the basis of the F.R.A. volume (separately determined), hydrogen concentration and the volume of air inspired (corrected to 37° C., with a constant deduction of 0·175 l. for subject's dead space). A number of paired experiments were performed with normal and with maximal inspirations. They are tabulated in Table 1.

Inspiration of hydrogen. These experiments were all performed on a single subject. At first they were carried out without preliminary oxygen breathing. The seated subject, quietly breathing air, applied himself to a mouthpiece (dead space 0·04 l.) and at the end of a normal expiration switched to a Benedict-Roth spirometer and took from it a measured inspiration of hydrogen of 99–100% purity. A subsequent alveolar sample was collected, and its hydrogen content estimated by the katharometer.

The transient anoxia resulting, especially from the larger inspirations, caused no subjective inconvenience. However, it was appreciated that the effect of even a relatively small inspiration of hydrogen on oxygen and CO_2 tension-gradients across the alveolar membrane—diminishing one and increasing the other—would be slightly to increase the alveolar air volume by raising the R.Q., thus there would be a tendency towards dilution of the alveolar hydrogen. Further experiments were therefore done, as before, but with preliminary oxygen-breathing, thus ensuring an adequate alveolo-capillary oxygen gradient throughout the experiment.

Experiments using nitrogen

When the nitrogen content of the F.R.A. is sufficiently lowered by preliminary breathing of a high oxygen mixture, then a subsequent inspiration of pure nitrogen becomes in effect an inspiration of foreign gas and the alveolar distribution may be followed with the same facility as that of hydrogen. An additional advantage is an accuracy of analysis of 0·05 % using the Sleigh modification of the Haldane gas analysis apparatus (Sleigh, 1937).

The apparatus was arranged as in the first group of hydrogen experiments (inspiration of air into alveolar hydrogen air mixtures). Circuit *A* was completely filled—after four or five preliminary rinsings—with pure oxygen. The subject was switched into circuit at the point of maximum expiration so as to introduce the minimum quantity of nitrogen. He then breathed quietly for 4 or 5 min. with the volume kept constant by an inflow of oxygen. The subject then detached himself from the circuit at the F.R.A. level or above (the exact point could be read from the spirometer tracing) and, with or without a preliminary complete expiration and alveolar sampling, drew a measured inspiration—the maximum possible from the starting level—of nitrogen, from circuit *B*. The subsequent expiration terminated with the collection of an alveolar air sample. When the nitrogen inspiration was taken from the resting respiratory level or above, a sample from the equilibrium mixture in the first spirometer was regarded as representative of the initial alveolar nitrogen content; otherwise, expiration and sampling preceded the nitrogen inspiration. Direct comparison revealed no disagreement between samples taken in these ways.

RESULTS

Table 1 gives the results of diluting intrapulmonary mixtures of air/hydrogen with air. There is with both small and large inspirations a measure of agreement between observed and predicted values for hydrogen content as close as can reasonably be expected.

TABLE 1. Hydrogen Dilution experiments

$$\text{Predicted final alveolar } H_2\ \% = \frac{\text{Equilibrium } H_2\ \% \times \text{F.R.A.}}{\text{F.R.A.} + \text{volume inspired}}$$

Subject	Equilibrium H_2 %	Volume air inspired (l.) (0·175 l. dead space to be deducted)	Alveolar H_2 %		Differences
			Observed	Predicted	
W.M.A. (F.R.A. = 4·66 l.)	6·26	0·830	5·30	5·49	−0·19
	6·16	3·150	4·00	3·76	+0·24
	6·17	0·715	5·47	5·53	−0·06
	6·10	2·260	4·53	4·22	+0·31
G.H.A. (F.R.A. = 3·57 l.)	6·80	0·810	5·72	5·77	−0·05
	6·65	3·120	3·55	3·64	−0·09
	12·45	0·860	10·50	10·44	+0·06
	12·20	2·350	7·36	7·58	−0·22
	12·45	1·050	10·00	9·99	+0·01
	12·45	2·430	7·50	7·63	−0·13
H.G.C. (F.R.A. = 2·52 l.)	12·65	0·950	10·00	9·67	+0·33
	12·50	2·850	6·25	6·06	+0·19

The results of hydrogen inhalations are plotted graphically in Fig. 1. A statistical analysis was made by listing the extent to which each observation differed from the predicted value. The mean of this unique sample of differences did not differ significantly from zero, the value of P being $<0{\cdot}2$ and $>0{\cdot}1$ (Fisher, 1944).

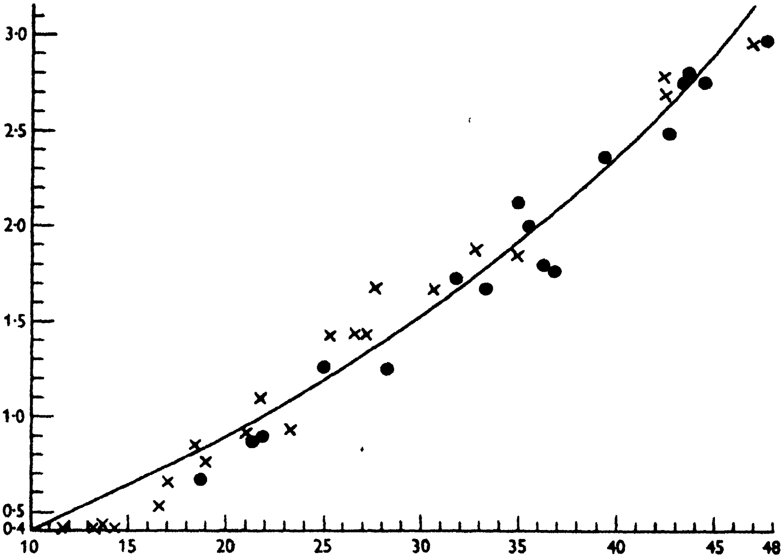

Fig. 1. Inspirations of H_2 from resting respiratory level. Comparison of observed with predicted values for alveolar H_2 concentration. ● observed concentration with preliminary O_2 breathing; × observed concentration with preliminary air breathing. Curve represents concentrations predicted from formula. Abscissa: percentage H_2 in final alveolar sample. Ordinate: volume of H_2 inspired (l.). Subject: G.H.A.

The fact that the hydrogen concentrations with small breaths tend to be greater than the predicted values suggests that the dead space may be exerting at these levels an influence which disappears with larger breaths. This aspect is more fully considered in a later section of the paper.

TABLE 2. Experiments with inspiration of N_2 to maximum capacity from various initial levels. (Subjects breathing O_2 beforehand)

Subject	Initial lung volume (l.)	Initial alveolar N_2 %	Volume (l.) inspired (dead space of 0·175 l. deducted)	Inspired N_2 %	Observed final alveolar N_2 %	Predicted final alveolar N_2 %	Differences
W.M.A.	2·66	20·30	4·15	96·90	66·20	66·97	−0·77
W.M.A.	4·66	21·19	2·86	99·42	53·40	50·96	+2·44
G.H.A.	1·54	19·48	4·12	99·24	76·10	77·53	−1·43
G.H.A.	3·57	16·27	2·52	99·14	51·08	50·55	+0·53
G.H.A.	3·57	10·91	2·70	98·70	47·91	48·71	−0·80
G.H.A.	4·69	15·43	1·45	99·56	34·05	35·29	−1·24
G.H.A.	4·94	15·49	1·47	97·91	35·51	34·39	+1·12
G.H.A.	5·67	15·55	0·60	99·61	22·91	23·58	−0.67

Inhalations of nitrogen give data which are tabulated in Table 2, together with the deviation of each from the predicted value. The overall result of these experiments is to confirm the hydrogen observations in suggesting that no significant operation of 'reserve alveoli' is involved in the taking of a deep breath.

Fractional analysis of expired air

The foregoing experimental results seemed clearly against the occurrence of important alterations in alveolar ventilation pattern in deep as compared with quiet breathing. This conclusion conflicted with no established opinion. But a second more controversial consequence was inherent in the same results, for these appeared to suggest that alveolar ventilation was uniform. This was contrary to the conceptions of Krogh & Lindhard (1917) on the one hand, and Sonne (1934) and Roelsen (1938 and 1939) on the other; for they, by the application of fractional sampling techniques, had independently shown that end-expiration alveolar samples almost always contained proportionately the least admixture of the preceding inspiration. These workers of otherwise divergent views were united in accepting this as evidence of relative under-ventilation of the deeper alveolar air; and unless the present findings could be reconciled with theirs any conclusion from them would be suspect. The remaining section represents an attempt at such reconciliation.

Single expirations are passed through a fractional sampler which collects five to six successive small aliquots, the bulk of the expiration passing into a recording spirometer. The collection of each sample actuates a signal on the spirometer tracing enabling the correlation of the sample with the phase of expiration (Armitage, Arnott & Pincock, 1949).

Preliminary experiments were made in which serial samples were taken from complete expirations following inspirations of hydrogen or helium. In general, the results obtained confirmed the Scandinavian reports in so far as a gradient was found indicating a higher concentration of the inspired gas in the earlier samples than in the later.

However, it still appeared true that the end-expiration samples were of the predicted composition; in other words, the observed gradient appeared to represent diminishing surpluses of inspired gas rather than a spread around a mean concentration found somewhere in the earlier part of the expiration, which is the concept implied in Roelsen's (1938) interpretation of similar findings.

The conclusion that a gradient of gas concentrations is indicative of unequal ventilation is supportable only if it can be shown that the later samples of an expiration have concentrations deviating as much in one direction from the level predictable on the assumption of uniform mixing as do the earlier samples in the other direction. In other words, in using these gas-dilution methods the basic requirement should be borne in mind that all the gas inspired must be accounted for in the expired and residual air; if higher values are found in some fractions then there must be correspondingly lower values in others so that the mean of the concentrations in all fractions equals the value that would result from uniform mixing.

It follows, therefore, that if a diminishing concentration of an inspired reference gas is not due to terminal contributions from imperfectly ventilated alveoli, then the earlier specimens must contain a surplus of reference gas derived from the dead space which is filled by the final portion of the inspiration. Through this massive concentration must pass the earlier fractions of the expiration, resulting in a heavy 'contamination' with a resulting content bearing no simple relationship to the concentration in the alveoli whence they came. In the use of the 'uniform mixing' formula to predict end-expiration concentrations this difficulty was overcome by deducting an assumed volume for the dead space from the volume inspired and therefore from the total volume of which the inspiration forms a part. This procedure is quite legitimate as regards an end-expiration sample as by the time it passes through the dead space the latter is washed clear of the inspired gas and contains a mixture very similar to that of the final sample.

The really important point is the relationship between the concentrations observed in the later samples and those predicted on the basis of uniform mixing. In the one example in which Roelsen (1938) makes this comparison the concentration in the final fraction is in fact some 2% lower than predicted, an observation at variance with the results reported earlier in this paper.

A close scrutiny of his results shows that the lung volume used in calculating the perfect mixing value is derived from the volume of hydrogen inspired divided by the alveolar concentration of hydrogen obtained by averaging the second to the fifth of the six fractional samples. Such a procedure makes it inevitable that the concentration in the final alveolar sample will be less than the predicted mean. It is clear that in order to make any useful deductions from the relationship of the 'uniform mixing' and final alveolar concentrations of a diluting gas it is essential that the estimation of the lung volume into which the inspiration is received must be made by an independent method which is quite free from any possible error inherent in the fractional sampling technique. Our procedure avoids this fallacy as it depends upon the initial estimation of hydrogen or helium concentration in the lungs by breathing to equilibrium in a closed circuit.

A method of demonstrating as well as correcting for the contaminating effect of the dead space is to wash in a known volume of diluent gas with a known volume of lung gas mixture ('equilibrium mixture') so as to ensure that the early fractions expired will not pass through pure diluent gas but through a mixture of closely similar composition to that in the lungs. Of course—taking an oversimplified illustration—contamination of 500 c.c. of alveolar air containing say 20% reference gas by passage through a dead space of 200 c.c. of air which it washes out by 50% will only reduce the reference gas concentration to 16%, whereas expiration through the same dead space filled with pure reference gas will raise its concentration to 33%. Therefore the procedure of completing the test inspiration of reference gas with air cannot be expected to alter the predicted concentration to the same extent as expiration through pure reference gas will elevate it, unless alveolar concentrations are of the order of 50%. Although this 'washing-in' procedure is crude it is capable of determining in a qualitative way the influence of the dead space on the successive fractions of an expiration.

METHODS

The arrangement of apparatus was very similar to that employed in the first section of this report with the addition of the fractional sampler and a three-way valve, so that switching from the closed to the open circuit could be effected without change of mouthpieces as in the earlier experiments. Helium instead of hydrogen was used as the reference gas. Inspiration of air into lungs filled with a helium/air mixture has the very substantial advantage of enabling an exact measurement to be made of the lung volume into which the air inspiration is taken. Furthermore, this arrangement is much more economical of the expensive gas helium than inspirations of pure helium into lungs filled with air. The helium content of these samples was measured in the katharometer after removal of CO_2 by bubbling through caustic soda and lime water. A portion of the sample was used for determination of the CO_2 content by the Haldane method. The helium percentage was corrected for the removal of the CO_2.

In some experiments the inspiration of air from spirometer *B* was washed in by a quantity of air/helium mixture from spirometer *A*.

RESULTS

The results of one experiment are reported in detail, each stage in the calculation being marked by a reference letter to simplify tabulation of the results of the group (Table 3).

Subject: W.M.A.

Large inspiration of air into lungs filled with air/helium.

Air *not* washed in with air/helium mixture.

Helium percentage in lungs in equilibrium with spirometer '*A*' = 6·45% (*a*).

Volume of mixture in lungs at instant of switching to spirometer '*B*' = 3·75 l. (*b*).

Volume of air inspired (less 0·175 l. for anatomical dead space and less 0·045 l. for dead space of mouthpiece and 3-way valve) = 2·05 l. (*c*).

Volume of air/helium mixture used to wash in air inspiration = Nil. (*d*).

Total volume in lungs at end of inspiration = 5·80 l. (*e*).

Predicted helium concentration assuming uniform mixing $\frac{6{\cdot}45 \times 3{\cdot}75}{5{\cdot}80}$

$= 4{\cdot}17$ (*f*).

Fractional analysis of expiration.

Sample	Litres expiration (*A*)	% He (*B*)
1	1·00	3·50
2	2·44	4·03
3	3·42	4·13
4	3·80	4·20
5	4·02	4·19

If 1 l. of 4·17 % air/helium mixture passes through dead space air and emerges as 1 l. of gas containing 3·50 % helium, then the amount of air/helium mixture which has been exchanged for air to effect this alteration is

$$1{\cdot}00-\left(1{\cdot}00\times\frac{3{\cdot}50}{4{\cdot}17}\right)=1{\cdot}00-0{\cdot}840=0{\cdot}160\text{ l.}$$

Making this calculation for each of the five fractions and summing the results the total surplus of air in the expiration becomes 0·216 l.

This surplus is derived from a total dead space of 0·300 l. made up of the dead space quoted above plus the additional dead space between the 3-way valve and the fractional sampling point. The calculation is very approximate as the figure for helium concentration is from the end of each fraction and this introduces a considerable error particularly in the first sample. There does not seem to be any simple method of interpolating the mean concentration of this first sample owing to ignorance of the mean velocity of the gas stream and in any case the impossibility of applying any mathematical treatment to flow through a tube of such complex internal configuration as the anatomical dead space.

TABLE 3

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)		1	2	3	4	5	'Surplus'
G.H.A. 'Control'	6·13	3·56	—	2·08	5·64	**6·13**	*A*	0·89	1·87	2·68	3·15	3·53	0·058
							B	5·91	6·00	6·08	6·08	**6·07**	
W.M.A. 1	5·50	4·59	**0·62**	–	5·21	**4·85**	*A*	0·68	1·74	2·36	2·60	—	0·189
							B	4·41	4·49	4·56	**4·75**	—	
W.M.A. 2	6·45	3·75	**2·05**	—	5·80	**4·17**	*A*	1·00	2·44	3·42	3·80	4·02	0·216
							B	3·50	4·03	4·13	4·20	**4·19**	
W.M.A. 3	5·65	4·03	**2·49**	—	6·52	**3·49**	*A*	0·31	1·69	3·40	4·15	4·40	0·346
							B	3·07	3 17	3·19	3·39	**3·30**	
G.H.A. 1	6·50	3·01	**1·56**	—	4·57	**4·28**	*A*	0·85	2·25	2·95	3·19	—	0·195
							B	3·83	4·03	4·14	**4·26**	—	
G.H.A. 2	14·21	3·25	**2·06**	—	5·31	**8·70**	*A*	0·59	1·68	2·48	2·92	3·48	0·208
							B	7·51	8·14	8·29	8·44	**8·61**	
C.T.G.F. 1	7·08	3·14	**1 65**	—	4·79	**4·64**	*A*	0·32	0·66	0·99	1·60	2·28	0·298
							B	2·75	3·94	4·12	4·25	**4·44**	
W.M.A. 4	6·20	3·35	**1·39**	0·38	5·12	**4·52**	*A*	0·40	1·18	1·70	2·11	2·70	—
							B	4·61	4·69	4·72	4·64	**4·56**	
G.H.A. 3	7·00	3·12	**0·86**	0·72	4·70	**5·72**	*A*	0·56	0·78	1·40	2·10	2·79	—
							B	5·68	5·81	5·80	5·82	**5·77**	
G.H.A. 4	6·72	3·23	**0·64**	0·42	4·29	**5·72**	*A*	0·90	1·60	2·09	2·66	2·85	—
							B	5·20	5·27	5·39	5·46	**5·52**	
G.H.A. 5	6·00	3·17	**1·31**	0·69	5·17	**4·48**	*A*	1·13	1·81	2·53	3·07	—	—
							B	4·31	4·29	4·36	**4·38**	—	

As the main theme of the paper is a study of mixing in a large inspiration, in Table 3 are emphasized the critical observations which show close agreement between observed values and those predicted on the basis of uniform mixing, irrespective of the size of inspiration and irrespective of whether or not the inspiration of diluting gas (air) is washed in by the same mixture (air/helium) as fills the lungs prior to the test inspiration.

It is clear from Table 3 that there is a slight diminishing gradient of diluting gas concentration (air) to the final sample when no washing-in is done, but the fact that the final concentration does not fall below the uniform mixing value suggests that this gradient is due to a surplus of air which can be derived only during expiration through the air-filled dead space. Calculation of the excess air represented by the gradient (although only very approximate) gives values compatible with such an origin. In this respect the control experiment in which no air is inspired gives a very slight gradient with an air surplus of only 58 c.c. which seems dependent on the exposure of this expiration to contamination by air during passage through the fractional sampler dead space of 80 c.c. between the 3-way valve and the fractional sampling point. The last four washing-in experiments show, in general, an obliteration of the gradient, although there is no reversal for the reason stated in the introduction to the final section; furthermore, although the expiration now passes through the main dead space filled with air/helium there is still the sampler dead space of 80 c.c. filled with air.

DISCUSSION

Much work has been done on the intrapulmonary mixing of gases, a very full review of which has recently been provided by Rauwerda (1946). One of the earliest contributions was that of Gréhant (1864) who studied the distribution in expired air of inspirations of a known volume of hydrogen; he found the hydrogen content of expired air to be independent of the depth of expiration and concluded that there was uniform ventilation of the lungs. Siebeck (1911) found essentially similar results except that uniformity was only reached after the expulsion of approximately 400 c.c. of air. Krogh & Lindhard (1917), while investigating the size of the dead space, used inspirations of pure hydrogen and found that the last sample taken during expiration invariably contained least hydrogen and concluded that there was a definite but slight inequality in the mixing. Sonne (1934) and Roelsen (1938, 1939), using fractional sampling of expired air after inspirations of hydrogen, also found that the concentration fell steadily to the level of the end-expiration sample. On the other hand, Darling, Cournand & Richards (1944), on the basis of a study of the progressive washing out of lung nitrogen by inhalations of oxygen, conclude that alveolar ventilation is uniform in the majority of normal subjects.

There seems little room for argument that our results exclude the operation of any 'reserve alveolar' mechanism with large as opposed to normal breaths.

While the incidental evidence gleaned from fractional analysis confirms above-quoted reports in that it shows a gradient of diluting gas falling to a minimal value towards the end of expiration, nevertheless, it is considered that this is not necessarily evidence of unequal alveolar ventilation but is an effect of the dead space. Darling *et al.* (1944) may have been thinking along similar lines when they point out the curious anomaly in Roelsen's results that when he used hydrogen, the most miscible gas of all, 'these experiments showed a greater degree of imperfect mixture than those using air (possibly) indicating ...that hydrogen diffused into stagnant air spaces not emptied by a forced expiration'.

In effect this means that the washing out of the dead space takes a great deal more gas than the variously estimated 300–700 c.c. While a volume of this order removes most of the dead-space gas it is maintained that a small portion remains which exerts a diminishing influence on subsequent samples—an influence which, although well nigh imperceptible when the difference is between 21 and 15% oxygen, is obvious when the dead space contains pure hydrogen.

We are engaged in applying these techniques to the ventilating problems of cardio-respiratory disease. The observations of Cournand, Baldwin, Darling & Richards (1941) on the much diminished rate at which the inhalation of oxygen can replace nitrogen in emphysematous lungs make it likely that a significant difference between observation and prediction will be found in that disease.

SUMMARY

1. In order to determine if voluntary large breaths are accommodated in the same alveoli used during quiet breathing the concentrations of hydrogen/nitrogen/helium and air in alveolar gas following inspirations of varying volumes of these gases were studied.

2. The observed concentrations did not differ significantly from those predicted on the assumption that the large breaths entered the same alveoli.

3. Fractional sampling revealed a gradient of inspired gas concentration falling to the level predictable on the basis of uniform mixing as expiration ended.

4. The magnitude of the surplus of inspired gas represented by this gradient is compatible with the assumption that it is derived during expiration from the dead space air (that is, a large expiration is needed to wash out the dead space).

We gratefully acknowledge the help and loan of a katharometer from Dr G. A. Shakspear, the receipt of an expenses grant from Messrs Imperial Chemical Industries Ltd., the assistance of Dr A. G. W. Whitfield, himself engaged on cognate problems, and the never-failing skill and industry of A. C. Pincock, Senior Technician to the Department.

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THE RENAL RESPONSES OF INFANTS AND ADULTS TO THE ADMINISTRATION OF HYPERTONIC SOLUTIONS OF SODIUM CHLORIDE AND UREA

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Of all the aspects of renal function which have been investigated in the last 20 years, the excretion of minerals is one of the least understood. This is partly because the kidney is not the only organ concerned, and partly because, even if it were, its treatment of chlorides, bicarbonates, sodium and potassium must inevitably be more complicated than its treatment of a substance such as inulin or even diodone. The response of the adult kidney to dehydration and to the administration of hypertonic solutions of sodium chloride and urea has been studied by various people, but few of the many problems have been solved.

Infants have much lower clearances of sodium and of chloride than adults, although the concentration of these ions in their serum is the same. It is also known that the newborn baby passes a very dilute urine *in utero*, and that even during the physiological hydropenia of the second and third days of life the urine is not nearly so concentrated as it would be under similar circumstances in adult life (McCance, 1948). It seemed desirable therefore to compare the reactions of the newborn and the adult kidney to the administration of hypertonic solutions in order to find out more of the ways in which the kidney of the infant differs from that of the adult, and to explore the mechanism underlying osmotic control at all ages. A summary of some of the findings was given by Dean & McCance (1947*a*).

The work now to be described has been done on babies with inoperable meningomyeloceles with the full consent of those responsible for their care. The practice has been for R. F. A. Dean to explain to each mother that it was hoped with her permission to make a test on her baby similar to ones which had already been made on healthy adults. She was assured that the work would not cause pain or harm, and that the information gained would benefit other children, although it could not cure her own child. Permission was always freely given.

METHODS

All the subjects were deprived of fluids for 15 to 16 hr. before the experiments. From adults, urine was collected for a measured time—usually about 2 hr.—before the saline or urea was administered. During the tests they reclined in comfortable chairs or on couches, standing up to pass urine. They were not catheterized and, except in a few instances when slight irregularities of minute volume were found, seemed able to empty their bladders satisfactorily. In the salt experiments, a 10% solution of NaCl was infused by gravity through a 'drip' connexion into one of the veins in the ante-cubital fossa; a cannula with a sharp trocar, which could be withdrawn later, was used. The blood samples were taken from the veins of the opposite arm, the syringes being well lubricated with liquid paraffin, and the blood was immediately centrifuged under paraffin. Heparin, or a mixture of potassium and ammonium oxalates, was used to prevent coagulation.

The first step in an experiment on an infant was to catheterize it. Then, and whenever a specimen was taken, its bladder was emptied as completely as possible with the aid of pressure on the abdomen. The backward end of the catheter was led into a small graduated measuring cylinder; the forward end remained in the bladder throughout the experiment. The resting urine was collected over a period of about 4 hr. The 10% NaCl solution was given by the 'drip' method through a cannula in an internal saphenous vein at the ankle. Blood was taken by needle and syringe through the anterior fontanelle with the precautions already described against exposure to air.

TABLE 1. Age, sex and factor for conversion of surface area of the subjects to 1·73 sq.m.; amount of sodium chloride administered and its effect on the blood chlorides

						Plasma Cl (m.equiv./l.)	
Name	Age	Sex	Factor	Dose (g./kg.)	Time taken to give dose (min.)	Before giving NaCl	Maximum
Ro.	Adult	♂	1·24	1·21	105	101	135
Wo.	Adult	♂	0·82	1·03	187	101	141
Wh.	Adult	♀	1·33	1·02	181	102	132
Pe.	Adult	♂	0·92	0·91	65	106	133
B.P.	4 days	♂	7·9	1·43	99	97	174
B.B.	3 days	♀	6·6	1·39	79	112	157
F.L.	8 days	♂	8·0	2·01	125	94	144
M.P.	3 days	♀	9·7	1·14	73	113	154
A.J.B.	5 days	♂	8·0	0·88	83	87	132

Urea was given by mouth as a 50% solution, to the adults from a glass and to the infants through a stomach tube.

The doses of NaCl and urea were calculated on the basis of body weight for they were intended to produce a comparable increase in the plasma at either age, but the infants received slightly more than the adults. The doses and their effects, with other details, are recorded in Tables 1 and 2.

TABLE 2. Quantities of urea given and the effects on the blood urea

			Plasma urea (m.mol./l.)		
Name	Dose (g./kg.)	Time taken to give dose (min.)	Before giving urea	Maximum	Time taken to reach maximum (min.)
Ro.	1·83	10	5·0	25	150
Wo.	1·43	2	5·7	33	240
Wh.	1·86	30	4·7	29	180
Lo.*	1·60	130	4·5	30	60
M.P.	1·82	5	4·4	36	240
A.J.B.	1·86	5	5·0	35	150

* This subject whose name does not appear in Table 1 was an adult female; the factor for the conversion of her surface area is 1·07. She and Ro. each vomited part of the dose.

One adult and two infants were given inulin in order that their glomerular filtration rates might be measured. The inulin was given to Pe., intravenously in 10% solution through the drip connexion. A priming dose of 25 c.c. was followed by a maintenance flow of 0·5 c.c./min. His glomerular filtration rate was measured before and after the salt had been given. The infants were given 10–20 c.c. of a 10% solution of inulin in 0·9% NaCl subcutaneously, over a period of about 5 min. into the loose tissues of the thigh.

The 'start' of each experiment was reckoned from the mid-point of the period of administration of NaCl or urea. The duration of urine collection tended to be long when the minute volume of urine was small and short when it was large. Blood was taken before, at intervals during, and at the end of the experiments. Curves drawn from the data obtained were used for calculating the concentration in the blood at the times required. The concentrations of the various specimens of urine were also plotted against time, making it possible to calculate the concentrations and clearances at 30 min. intervals throughout the experiments.

The methods used for the chemical analysis were as follows: Chlorides—Whitehorn's (1920) method for the adult urines; the method of van Slyke (1923–4) as modified by McCance & Shipp (1933) for the plasma of the adults; Sendroy's (1937) iodine titration method for the urines and plasma of the infants. Urea—the methods of Lee & Widdowson (1937) and Archibald (1945). Inulin—Roe's (1934) method, as modified by Cole (to be published). In presenting the results all the minute volumes have been calculated to a standard basis of surface area which has been taken to be that of a 70 kg. man (1·73 sq.m.). The 'correction' factors are given in Tables 1 and 2. Further information about methods and chemical technique may be found in the paper by Dean & McCance (1947*b*).

RESULTS

Sodium chloride experiments

The plasma chlorides. The administration of large doses of NaCl naturally raised the Cl concentration in the plasma. In adults the highest value was obtained soon after the salt had been given. Thus Ro.'s plasma Cl rose from 101 to 135 m.equiv./l. within 30 min. of the 'start', and thereafter fell very slowly so that it was still 130 m.equiv./l. 240 min. later. In the infants the initial rise was similar, but a small secondary or further rise sometimes took place long after the administration of NaCl had ceased. Thus A.J.B.'s plasma Cl was 87 m.equiv./l. before the experiment began. After 60 min. from the 'start' it was 126 m.equiv./l., and 180 min. from the start it was 131 m.equiv./l. It was still 127 m.equiv./l. after 390 min. Apart from the small secondary rise in the infant's plasma Cl the changes were comparable and similar at both ages and it is not thought that the differences between adults and infants which have been revealed by a study of the urines can be explained by the differences in the composition of the plasmas.

The greatest urine flow did not necessarily coincide with maximum concentration of Cl in the plasma. Thus the adults Pe. and Wh. had their maximum diuresis between the 60th and 90th min. at a time when their plasma Cl were falling. The infant M.P.'s maximum diuresis occurred between the 180th and 210th min., but the maximum plasma Cl was not reached till the 270th min. In the infants B.B. and A.J.B., and in the adult Ro. the two maxima coincided.

The minute volumes. Fig. 1 shows the effect on the minute volumes of administering 10% NaCl in the way described. The results of all the adults and of the infants were averaged for the construction of this figure. The adults' diuresis was rapid in onset, intense while it lasted but short-lived; the infants' diuresis was slow to commence, slight in intensity, but much more prolonged. The production of a diuresis in this way has frequently been demonstrated before in adults but less attention has been directed to the fall in urine flow which takes place while the plasma is still grossly abnormal. Some failure of NaCl to produce a diuresis in infants might have been anticipated from the work of McCance & Wilkinson (1947) on newborn rats, in which no increased flow of urine followed the administration of hypertonic NaCl. The fact that some increased flow of urine has been observed in human infants suggests that their kidneys are further developed at birth than those of the rat.

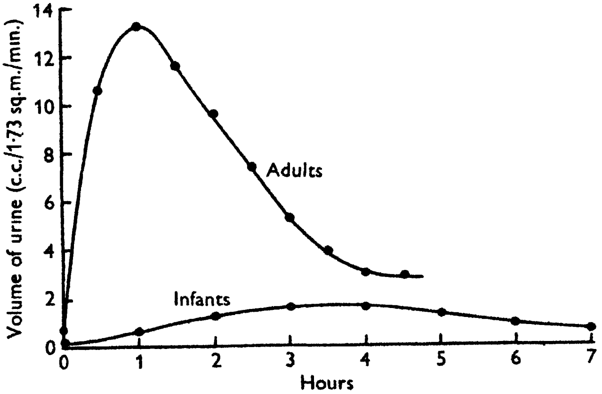

Fig. 1. The effect on the minute volume of the urine of giving hypertonic saline to adults and infants.

Table 3. The effect of giving 10% saline intravenously on the volume and composition of the urine of an adult and an infant

Time after saline was given (min.)	Pe. (adult)				B.B. (infant)			
	Min. vol. (c.c./min./1·73 sq.m.)	Urea (m.mol./l.)	Cl (m.equiv./l.)	O.P. (m.mol./l.)*	Min. vol. (c.c./min./1·73 sq.m.)	Urea (m.mol./l.)	Cl (m.equiv./l.)	O.P. (m.mol./l.)*
Pre-experiment	0·62	467	185	837	0·23	83	31	145
30	11·00	38	252	542	0·28	78	38	154
60	18·00	30	248	526	0·70	78	106	290
90	19·10	26	239	504	1·12	75	173	421
120	17·00	28	233	494	1·56	71	194	459
150	13·00	35	236	507	1·93	67	205	476
180	8·80	50	253	556	2·14	63	212	487
210	6·08	78	277	632	1·95	64	214	492
240	4·45	99	283	665	1·66	69	216	501

* Reckoned as m.mol. urea + 2 × m.equiv. chloride.

The composition and osmotic pressure of the urine. It was known to Starling (1909), probably from the work of Dreser (1892) and Galeotti (1902), that the administration of hypertonic saline caused a diuresis during which the osmotic pressure of the urine fell. This was shown to apply to man by McCance & Young (1944), McCance (1945) and Hervey, McCance & Tayler (1946*a*, *b*). The figures of Table 3 have been taken from the experiment on Pe. (an adult) and B.B. (an infant) and are characteristic of the present series. The concentrations of urea and of Cl in the initial urine of the adult were high, as was to be expected on a normal diet after 15 hr. without water. The diuresis which took place greatly reduced the level of urea in the urine, and the lowest figure observed was only one-eighteenth of the initial one. The concentration of Cl started at 185 m.equiv./l., varied between 248 and 233 m.equiv./l., while the flow of urine was very fast and then gradually rose to 283 m.equiv./l. (1·72 g. of NaCl/100 c.c.) over the next few hours. The concentration of Cl attained was, at the height of the diuresis, only 1·3 times and at the end 1·5 times the initial one. By contrast the concentration of urea in the infant's pre-experimental urine was only 83 m.mol./l. and the Cl 31 m.equiv./l. The other infants had rather higher concentrations of urea in their 'resting' urines, the highest figure being 148 m.mol./l. The concentration of Cl was, however, a representative one, the figures for the other infants varying from 9 to 34 m.equiv./l. Since the flow of urine increased much less in B.B. than in Pe. the concentration of urea in the urine fell correspondingly less. The concentration of Cl, however, rose slowly to 216 m.equiv./l. Thus, although the concentration was 7 times that at the start, it was never much higher than Pe.'s had been at the beginning. The effect of these changes in concentration were that the osmotic pressure of the adult's urine fell from its high resting value as the diuresis developed, whereas that of the infant rose from a relatively low starting level, but never attained a value appreciably greater than that of the adult during the height of his diuresis.

Osmotic work. In some of these experiments a calculation has been made of the osmotic work involved in altering the concentrations of NaCl and urea in the plasma to those found in the urine. The formula used has been modified from that of von Rhorer (1905). The results obtained probably represent over 80% of the total osmotic work required for the elaboration of the complete urine (Borsook & Winegarden, 1931), and may be taken as a rough measure of it. Fig. 2 shows the data obtained on the adult Pe. and the infant B.B. It can be seen that the osmotic work carried out per min. varied enormously and, roughly speaking, followed the minute volumes, so that before the saline was administered the tubules were capable of carrying out much more osmotic work per min. than they were doing, and there is no evidence that the tubules were ever exerting their full capacity even at the height of the diuresis. This makes the explanation of Hervey *et al.* (1946*a*, *b*) of the fall in the osmotic pressure of the adult urine with the onset of the diuresis rather inadequate for they supposed that the amount of osmotic work carried out by the tubules/min. during such a diuresis was approximately constant and of the same order of magnitude as that being carried out before the diuresis began. Yet some limitation of the capacity of the tubules to do the necessary osmotic work per unit of time seems the most satisfactory way of explaining the fall in the osmotic pressure of the urine.

Fig. 2 also shows that although the osmotic work carried out per min. by adults greatly exceeds that carried out by infants, the amount carried out per c.c. of urine formed is of the same order at both ages.

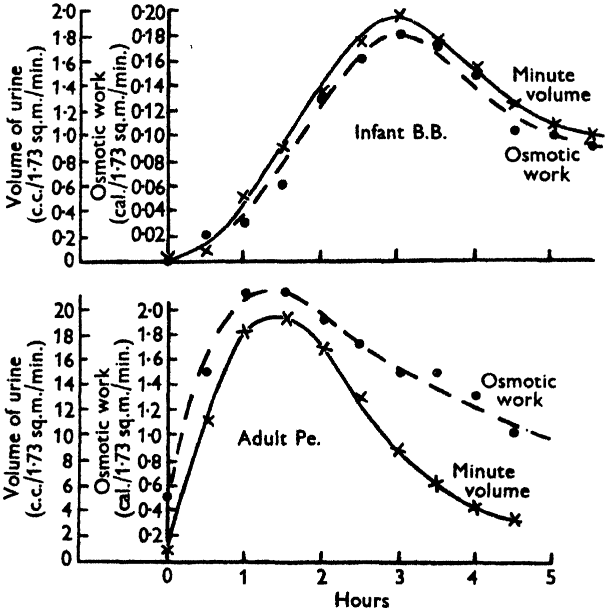

Fig. 2. Changes in the osmotic work of the kidney and in the minute volume of the urine after the administration of hypertonic saline to the infant B.B. and the adult Pe. Note that the scale for the infant's osmotic work and the urine volume is 1/10 that of the scale for the adult's.

Glomerular filtration rate. It has been shown by Dicker (1948) that the administration of hypertonic saline to rats raises their glomerular filtration rates and Mokotoff, Ross & Leiter (1948) used hypertonic saline to raise the glomerular filtration rates in their experiments on men. These papers had not been published when the experiments now being described were made. Table 4 shows our results. No figures were obtained for the glomerular filtration rates of the two infants before any saline had been given, but it will be seen that in the adult Pe. when the diuresis was at its height the rate was more than twice the accepted figure for normal people, to which figure (120–130 c.c./min.) it returned when the urine was still being formed at the rate of nearly 13 c.c./min. It is clear also that the saline greatly raised the glomerular filtration rates of both the infants, but the rise took place more slowly than in Pe. and at no time did the rate approach that of the adult. The comparison brings out once more the apparent inefficiency of the kidney in infancy and its slow response to a change in the internal environment. The alterations in urine flow must have followed to some extent the rise and fall in the glomerular filtration rate, but it appears that the flow of urine was much more subservient to the glomerular filtration rate in infancy than in adult life, for the relationship was much closer in the infants than in Pe. These experiments offer a possible explanation of the fact that some people have found that the glomerular filtration rates in infancy vary with the minute volume (Barnett, Perley & McGinnis, 1942; Young & McCance, 1942) while others have not (Barnett, McNamara, Hare & Hare, 1948). The result may easily depend upon whether the fluid used to induce the diuresis was intravenous saline, water by mouth or intravenous glucose solution.

TABLE 4. The effect of hypertonic saline on the minute volumes and the inulin clearances (glomerular filtration rates) of the adult Pe. and the infants B.P. and B.B.

	Pe. (adult)		B.P. (infant)		B.B. (infant)	
Time after mid-point of giving saline (min.)	Min. vol. (c.c.)	Inulin clearance (c.c./min./ 1·73 sq.m.)	Min. vol. (c.c.)	Inulin clearance (c.c./min./ 1·73 sq.m.)	Min. vol. (c.c.)	Inulin clearance (c.c./min./ 1·73 sq.m.)
	0·63*	127*	—	—	—	—
30	—	—	0·37	6·5	0·28	2·85
60	18·00	293	0·85	14·1	0·70	7·42
90	19·10	286	2·28	19·4	1·12	12·3
120	17·00	208	2·30	20·0	1·56	17·0
150	13·00	131	1·84	19·4	1·93	18·8
180	8·80	131	1·48	16·9	2·14	20·5
210	6·08	120	1·20	14·2	1·95	19·0
240	4·45	132	1·00	11·9	1·66	20·0
270	—	—	—	—	1·42	16·9
300	—	—	—	—	1·28	11·7

* Measured before any saline had been given.

The reabsorbates. Table 5 shows the glomerular filtration rates, the percentages of water and chloride in this filtrate which were reabsorbed in the tubules, and the m.equiv. of Cl reabsorbed/100 c.c. of glomerular filtrate. The inulin plasma clearances were taken as measuring the glomerular filtration rate, although it would probably have been more correct to have taken $I.c. \times \frac{100-x}{100}$, where $I.c.$ = the inulin plasma clearance and x = the percentage of protein in the plasma. The percentage of that filtrate which was reabsorbed was calculated by deducting from it the minute volume of urine. The Cl reabsorbed per min. was calculated as the inulin plasma clearance per min. × concentration of Cl in plasma × 1·05 (a correction introduced because of the impermeability of the glomerular membrane to protein) – Cl excreted per min. It will be seen that the percentage of the filtrate water reabsorbed by Pe. was 99·5 before the experiment began and fell with the onset of the diuresis, so that at first the large increase in the flow of urine was partly due to an increase in the volume of fluid filtered off in the glomeruli per minute, and partly to a decrease in the per-

TABLE 5. The amounts of filtrate water and chlorides reabsorbed by the tubules

	Pe. (adult)				B.P. (infant)				B.B. (infant)			
			Cl reabsorbed				Cl reabsorbed				Cl reabsorbed	
Time from start (min.)	Glomerular filtration rate (c.c./min.)	Percentage of filtrate water reabsorbed	As percentage of filtered Cl	(m.equiv./100 c.c. of glomerular filtrate)	Glomerular filtration rate (c.c./min.)	Percentage of filtrate water reabsorbed	As percentage of filtered Cl	(m.equiv./100 c.c. of glomerular filtrate)	Glomerular filtration rate (c.c./min.)	Percentage of filtrate water reabsorbed	As percentage of filtered Cl	(m.equiv./100 c.c. of glomerular filtrate)
	127*	99·5*	99·0*	10·6*	—	—	—	—	—	—	—	—
30	—	—	—	—	6·5	94·0	96·0	16·6	2·8	89·4	97·5	15·6
60	293	93·8	88·8	12·3	14·1	93·6	93·5	17·0	7·4	89·6	93·5	15·3
90	286	93·2	88·0	12·1	19·4	88·6	87·6	17·1	12·3	91·0	90·0	15·0
120	208	92·0	86·0	12·0	20·0	87·0	86·6	16·4	17·0	90·5	89·5	15·1
150	131	90·1	82·7	11·6	19·4	89·5	88·2	15·8	18·8	90·0	87·5	15·1
180	131	93·2	87·0	11·9	16·9	90·4	88·2	14·8	20·5	88·5	86·5	15·0
210	120	95·0	89·0	11·9	14·2	90·8	88·5	14·1	19·0	88·6	87·0	15·1
240	132	97·0	93·5	12·0	11·9	90·8	87·7	12·8	20·0	91·5	89·0	15·1
270	—	—	—	—	—	—	—	—	16·9	91·7	89·0	14·8

* Measured before any saline had been administered.

centage of that filtrate reabsorbed. As the glomerular filtration rate fell, this percentage continued to decrease and reached 90, which was its lowest point, after the glomerular filtration rate had returned to normal. Thereafter it gradually rose again and thus the urine flow continued to fall. The two infants reabsorbed a somewhat smaller percentage of the water in the glomerular filtrate at most stages of the experiments than did Pe., and in the case of B.P. the percentage reabsorbed was at its lowest about the time of the greatest diuresis. The rise and fall in urine flow were due to the rise and fall in the glomerular filtration rate in both children, but only in B.P. to a fall and rise in the percentage of the filtrate reabsorbed.

The third columns of each section of Table 5 show the percentage of the filtered Cl which was reabsorbed. Owing to the glomerular filtrates having been more than doubled by the hypertonic saline, and the blood Cl considerably raised, much more Cl was passed down the tubules per min. The figure in Pe. rose from about 14 to 40 m.equiv./min., and yet in this subject the percentage of the filtered Cl which was reabsorbed fell but little during the experiment, and the amount of Cl reabsorbed rose from 10·6 to 12·3 m.equiv./100 c.c. of glomerular filtrate at the peak of the filtration rate. It fell to 11·6 as the latter returned to normal and subsequently rose again to 12·0. Had it not risen again the minute volume would no doubt have been larger towards the conclusion of the experiment. The infants reabsorbed 86·5–97·5 % of the filtered Cl after the saline had been given, but the figures over 90 % were confined to the first hour. They would certainly have been absorbing more than 97·5 % before the experiments began. In B.B. the Cl reabsorbed amounted to about 15 m.equiv./ 100 c.c. of glomerular filtrate throughout the experiment. In B.P. a figure of 17·1 was reached 90 min. after the start, and subsequently the amount reabsorbed gradually fell to 12·8 m.equiv./100 c.c. of glomerular filtrate.

The concentration of chloride in Pe.'s plasma water before the experiment began was 111 m.equiv./l. and almost exactly the same in the reabsorbate. The concentrations in the infants' plasmas were 102 and 117 m.equiv./l., but the concentrations in the reabsorbates before any saline had been given are unknown. They would probably have been slightly more than they were in the plasma. Table 6 shows the concentration of chloride in the serum water and in the fluid reabsorbed by the tubules at various stages throughout the experiments on Pe., B.P. and B.B. The concentration of chloride in the reabsorbate was never very far from that in the serum water and in Pe. it was always lower in the reabsorbate after the saline had been administered. In the infants, even when the concentration of chloride in the serum water had reached highly abnormal figures (Table 6) and had been at those levels for some time, the concentration of Cl in B.P.'s reabsorbate was equal to and in B.B.'s actually greater than that in the plasma. Only after 2 hr. had the relationship to be expected with a hypertonic plasma been established. This is another aspect of the slow renal adaptation of infants to changes in the plasma: even some time after all the hypertonic saline had been administered, the infants were still excreting a urine which contained a smaller concentration of Cl than plasma.

TABLE 6. The concentration of Cl in serum water and in the fluid reabsorbed by the tubules

	At 60 min.		At 120 min.		At 180 min.	
	In serum water	In reabsorbate	In serum water	In reabsorbate	In serum water	In reabsorbate
Pe. (adult)	142	132	137	126	136	128
B.P. (infant)	184	183	179	173	164	158
B.B. (infant)	157	162	164	158	167	159

All the data are given in terms of m.equiv./l.

Urea experiments

Since the urea was given by mouth, the rate and completeness of its absorption from the gastro-intestinal tract were of some importance in interpreting the urinary findings. Table 2 showed that blood values of the same order of magnitude were achieved in both the infants and adults, and that the maxima were attained at about the same time after the urea had been administered. It is justifiable to conclude that urea was absorbed at about the same rate at the two ages and that the urinary differences about to be described originated in the kidney itself.

Fig. 3 compares the results of the experiments on one adult, Wo. and on one infant, A.J.B. In both the urea was given quickly—see Table 2—and the blood concentrations behaved, on the whole, in a similar manner. With regard to the minute volumes of the two subjects it will be seen that, as after giving NaCl, the diuresis fell off while the plasma was still very abnormal. The differences between the two subjects are obvious. As in the salt experiments the failure of the hypertonic solution to produce any noteworthy diuresis in A.J.B. is the most obvious renal peculiarity of infancy. The concentration of urea in Wo.'s urine fell after the urea had been given (Fig. 3). A similar effect was observed by McCance & Young (1944) and is associated with the diuresis. There is, however, no obvious explanation of this at present since there seems to be no rigid limitation to the amount of osmotic work which the kidney can carry out

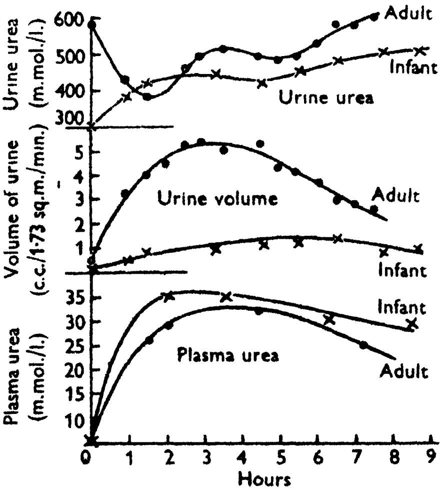

Fig. 3. Urine urea, urine volume, and plasma urea following the administration of hypertonic urea to the adult Wo. and the infant A.J.B.

per minute. The concentration of urea in the infant's urine rose as the blood urea rose, and between the 60th and 120th min. was as great as in the adult's. Thereafter Wo.'s urine became more concentrated as its flow diminished. In Wo. the diuresis and the high concentration of urea in the urine during the latter part of the experiment led to the excretion of a large part of the dose by the 460th min. (The other adults all had concentrations of urea over 670 m.equiv./l.) The comparative failure of the diuresis in A.J.B., coupled with his lower urinary concentrations of urea (in spite of higher serum levels) prevented him getting rid of much of his dose. The other adults responded much as did Wo., and M.P. behaved very like A.J.B., and a figure showing the average urea excretions was given by Dean & McCance (1947*a*). Table 7 shows the urea clearances and the urine osmotic pressures (reckoned as m.mol. of urea/l. + 2 × m.equiv. of Cl/l.) of Wo. and A.J.B. The low urea clearances of the infant confirm all previous work. An interesting feature, however, is the fall which took place in them after the urea had been administered. The clearance was not back at its initial level till the 108th min. The fall was due to the rapid rise in the concentration of urea in the plasma failing to increase the rate of excretion fast enough to maintain the clearance. In Wo. the initial clearance was high for a minute volume of 0·55, and it rose slowly in spite of the great increase in the blood urea. The rise of Wo.'s urea clearances was due to the diuresis which increased the output per min. as fast as the blood urea rose, in spite of the fall in the concentration of urea in the urine.

TABLE 7. The urea clearances after the administration of urea, and the urine osmotic pressures of an adult (Wo.) and an infant (A.J.B.)

	Wo. (adult)		A.J.B. (infant)	
Time (min.) after giving urea	Urea clearance (c.c./1·73 sq.m.)	Urine O.P. (m.mol./l.)*	Urea clearance (c.c./1·73 sq.m.)	Urine O.P. (m.mol./l.)*
Pre-experiment	49	961	11·1	414
45	53	573	7·9	474
90	60	562	8·7	508
150	78	608	10·4	516
240	76	634	13·3	522
330	66	606	15·5	535
450	61	703	16·5	555
600	—	—	14·6	575
660	—	—	13·1	569

* Reckoned as m.mol. urea + 2 × m.equiv. chloride.

The osmotic pressure of Wo.'s urine was high before the experiment, fell with the diuresis and began to rise again as the flow of urine subsided. This is the characteristic adult response to a sufficiently large dose of hypertonic urea—as it is to a similar dose of hypertonic salt (McCance & Young, 1944). Rabbits (Dreser, 1892), dogs (Galeotti, 1902) and rats (McCance & Wilkinson, 1947) give a similar response. The osmotic pressure of A.J.B.'s urine was low before the experiment began and rose steadily after the urea had been given, because

TABLE 8. Maximum concentrations of chloride and urea observed in the urines, the osmotic pressure of the urines, and the U/P osmotic ratios

	Saline experiments				Urea experiments			
		Urine				Urine		
Subject	Plasma Cl (m.equiv./l.)	Cl (m.equiv./l.)	Osmotic pressure (m.mol./l.)	Osmotic U/P ratio	Plasma urea (m.mol./l.)	Urea (m.mol./l.)	Osmotic pressure (m.mol./l.)	Osmotic U/P ratio
Ro. (adult)	134	285	652	2·4	22	732	834	3·7
Wo. „	141	272	618	2·2	24	598	703	3·1
Wh. „	125	346	792	3·1	23	683	767	3·2
Pe. „	124	286	679	2·7	—	—	—	—
Lo. „	—	—	—	—	19	720	884	4·0
B.P. (infant)	155	221	510	1·6	—	—	—	—
B.B. „	156	215	496	1·5	—	—	—	—
F.L. „	144	243	561	1·9	—	—	—	—
W.P. „	151	230	498	1·6	34	513	565	2·0
A.J.B. „	131	170	468	1·7	29	500	569	2·5

the concentration of urea was increasing in the urine and the diuresis was so small. This would appear to be the usual response of an infant.

DISCUSSION

A linkage of the chloride reabsorption to the glomerular filtration rate has been demonstrated by Lotspeich, Swan & Pitts (1947), and by Pitts & Lotspeich (1946) in adult dogs, and Mokotoff, Ross & Leiter (1948) found that the m.equiv. of Na reabsorbed by the kidneys of adult men also bore a constant relationship to the glomerular filtration rate. The figure given by them was 13·3 m.mol./100 c.c. of glomerular filtrate. The present experiments make it clear that these statements are approximately true, for in spite of great changes in the glomerular filtration rates in Pe. and in the two infants, the m.equiv. of Cl reabsorbed/100 c.c. of glomerular filtrate did not alter very much (Table 5). There were, however, fluctuations which have already been described. If one can distinguish cause and effect it would appear that the chloride reabsorbed/100 c.c. of glomerular filtrate is relatively constant because: (1) nearly all the water in the glomerular filtrate is always reabsorbed; (2) the concentration of chloride in the plasma water can only vary within relatively small limits; and (3) the concentration of chloride in the fluid reabsorbed cannot differ by very much from the concentration in the fluid filtered off. The variations which may be observed under normal or experimental conditions are due to the facts that: (*a*) the percentage of the filtrate water, which is reabsorbed, can vary from about 87 to 99·5%; and (*b*) the concentration of Cl in the plasma water can be made to vary by experiment from, say, 90 to 180 m.equiv./l. The variations introduced by this, see (2) and (3) above are, however, limited because the concentration of Cl in the reabsorbate tends to be lower than that in the serum when the latter is high, and higher than that in the serum when the latter is low. With some reservations, therefore, the findings of Lotspeich *et al.* (1947) have been shown to hold for an adult man, and evidently apply also to infants. It is interesting that both B.P. and B.B. reabsorbed more Cl/c.c. of glomerular filtrate than Pe., and it is evident that from this point of view the tubules of the infant's kidneys were in no sense inferior to those of adults. These experiments show that if the plasma chlorides are artificially raised the kidney does not, as was at one time supposed, reabsorb a fluid optimal for plasma composition, but only one which is a somewhat better approximation to the optimal than the fluid filtered off.

There has been some debate in the last 4 years as to the ability of an infant to produce a concentrated urine. The evidence was reviewed by McCance (1948), who came to the conclusion that when an infant was first deprived of water its urine was always much less concentrated than that of an adult treated in a similar way. If, however, the infant had been dehydrated for some time and particularly if the serum had become highly abnormal, then comparatively high osmotic pressures might be found in the urine. The present experiments provide some further evidence. Table 8 shows the maximum concentration of Cl and of urea observed in the urine during these experiments, the osmotic pressure of the urines, and the values of Cl or of urea in the plasma at the same time. It will be seen that under these abnormal conditions the infant urines had much higher osmotic pressures than is usual at that age (McCance & Young, 1941), and when the osmotic pressures in the plasma were calculated in the same way as those in the urine, U/P osmotic ratios of 1·5 to 2·0 were regularly found in the Cl experiments and one ratio of 2·5 in a urea experiment. The infant urines, however, never had such high osmotic pressures as those of the adults in whom the U/P osmotic ratios varied from 2·2 to 3·1 in the saline experiments and were all over 3·1 in the urea experiments. It is of some interest that the U/P osmotic ratios were always higher after urea than after saline administration, for Young, Hallum & McCance (1941) considered that a high blood urea was one of the things which might enable newborn infants to produce the high U/P ratios occasionally found by them. It is still a problem to what extent these concentrated urines in infancy are caused by the failure of the kidney to produce a diuresis at that age. If the diuresis produced by hypertonic solutions is due solely to the increase of osmotically active material delivered to the distal tubules (see later) it is difficult to see why these solutions do not cause a better diuresis in infants, and if osmotically active material in the distal tubules does not produce a diuresis in infants, one at once begins to wonder why infants do not always produce concentrated urines 5 or 6 hr. after being deprived of water.

Sodium chloride and urea have very different physiological properties, yet the administration of a strong solution of either of them to an adult produces very similar effects. These are (1) an immediate and often extensive increase in urine flow, which soon subsides, at first rapidly and then gradually, to a more usual rate, and (2) a fall in the osmotic pressure of the urine as the diuresis develops, to be followed by a rise to high levels as the flow of urine falls off. It is usually supposed that these diuretic effects are due to the excess of osmotically active material reaching the distal tubules. In infants, hypertonic solutions of sodium chloride and urea also produce effects which are very similar to each other, but these effects differ from those produced in adults, for (1) the diuresis develops and subsides very slowly and is always trifling in amount, and (2) the osmotic pressure of the urine tends to rise from the time the hypertonic solutions have been administered.

Before offering any explanation of these findings, it may be helpful to summarize some of the other ways in which the renal function of infants differs from or resembles that of adults. Thus: (1) the glomerular filtration rates are much lower as are the urea, phosphate, sodium and chloride clearances; (2) the percentage of the urea in the glomerular filtrate which reaches the urine is perhaps slightly lower in infants than in adults, but is roughly the same at both ages; (3) infants and adults reabsorb about the same percentage of the Cl in their glomerular filtrates; (4) infants reabsorb rather more Cl/100 c.c. of glomerular filtrate than adults; and (5) infants cannot produce urines with such high osmotic pressures as normal adults. The first of these points suggests that infants have very defective glomerular function, but the second, third and fourth that their tubular function is fully developed so far as urea and Cl are concerned, and that it is linked with the glomerular filtration rate as it is in adults. It is suggested, therefore, that it is the difference in glomerular function which must underlie the differences in adult and infant responses to hypertonic urea and NaCl. It is possible that *in utero* and for some time thereafter the glomeruli are outside the main renal blood flow, or it may be that the layer of cubical cells overlying the glomerular tufts prevents effective filtration. It is impossible at present to decide one way or the other.

Since infants cannot produce such concentrated urines as adults, it might be suggested that the distal tubules were unable to prevent the back diffusion of urea and Cl, and that this was the reason for the failure of the diuresis to develop. This, however, is not in keeping with observations 2, 3 and 4 above, and that being so, one would expect a failure of the distal tubules to concentrate the urine to increase rather than diminish the diuresis due to hypertonic solutions.

If the fall in the osmotic pressure of an adult's urine, following the administration of hypertonic solutions is due to the intense diuresis, then its absence in infancy is easily explained by the small, and perhaps also the slow, change in urine flow which takes place at that age.

A rise in glomerular filtration rate follows the administration of hypertonic saline. Like the urine flow the rise in the adult Pe. was short and violent, that in the two infants slow, prolonged and relatively somewhat greater. It would probably be very helpful to know the reason for these time-relationships, but it is not yet known why the hypertonic saline should raise the glomerular filtration rate as it undoubtedly does. The rise may be due, in part at any rate, to an increase in plasma volume, and consequently a decrease in plasma colloidal pressure, caused by the withdrawal of water from the cells (Eggleton, Pappenheimer & Winton, 1940). It seems certain, however, that these changes in glomerular filtration rate must underlie and in part determine the observed changes in urine flow.

SUMMARY

1. When hypertonic saline was administered to normal adults after 15 hr. without water: (*a*) the urine flow increased rapidly and extensively and then fell, at first rapidly but subsequently more slowly; (*b*) the glomerular filtration rate rose at the same time as the urine flow, but it had returned to normal long before the diuresis had subsided.

2. When hypertonic saline was administered to infants after the same length of time without water: (*a*) the urine flow increased slowly and to a very moderate degree, and fell again equally slowly; (*b*) the glomerular filtration rate rose and fell with the same time-relationships as the urine flow.

3. In these infants the reabsorption of Cl/100 c.c. of glomerular filtrate was appreciably greater than it was in adults.

4. When a strong solution of urea was administered to adults and infants the changes produced in the flow of urine were not so great as those produced by hypertonic saline, but resembled them in other respects. The mechanism is likely to be the same, but the glomerular filtration rate may not increase.

5. The diuresis produced in adults by hypertonic saline or urea was accompanied by a fall in the osmotic pressure of the urine with a subsequent rise to high levels as the diuresis subsided.

6. The administration of hypertonic saline or urea to infants was characteristically accompanied by a rise rather than a fall in the osmotic pressure of the urine, and it is suggested that this was due to the fact that the diuresis was so small.

7. Owing to their greater diuresis, and to the higher osmotic pressure of their urines, adults excreted a much larger percentage of the hypertonic saline or urea than infants—at all events within the time limits of the experiments.

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THE SUGAR OF THE FOETAL BLOOD, THE AMNIOTIC AND ALLANTOIC FLUIDS

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These observations were made in order to obtain quantitative knowledge of the distribution of fructose and glucose in the blood and in the amniotic and allantoic fluids of the sheep foetus at different ages.

In 1854 Claud Bernard identified fructose in cow's amniotic fluid by its laevo-rotation; Gürber & Grünbaum (1904) recorded it in the fluids of the sheep, cow and goat. Paton, Watson & Kerr (1907) found sugar in the fluids of the sheep, cow, pig, cat, dog, rabbit, ferret and guinea-pig, but identified it as fructose only in the sheep and cow. Their identification rested on (*a*) Seliwanoff's reaction, (*b*) laevo-rotation, (*c*) yeast fermentation, and (*d*) osazone formation with phenyl-hydrazine. They did not discuss the presence of glucose either alone or simultaneously with the fructose. They estimated the sugar by a method unstated and express it as 'G' in their tables, never specifically stating that it was estimated as fructose (it might have been estimated as glucose), always speaking of it as 'sugar'. Takata (1922) found fructose in the amniotic fluid of the whale and Orr (1923) first thought it to be present in human foetal blood. In 1927 van Creveld described a method of estimating fructose and van Creveld & Ladenius (1928) showed the results of fructose tolerance tests in pregnant women. The blood fructose concentration never rose above 14 mg.%. Yamada (1933) found fructose in chick's amniotic fluid and in the same year Okamura discussed the question of sugars other than glucose in the blood. Four years later he determined the total blood sugar of the pregnant woman as being between 67 and 150 mg.%, of which 2·2–5·0 mg. were fructose. This was in the period between the third and tenth lunar months, but there was no relation between concentration and age of pregnancy.

All this time there had been some doubt as to the identification of the blood sugar as fructose. Bacon & Bell (1946, 1948), however, not only successfully isolated this osazone but also obtained the diacetone derivative. At the same time Cole improved Roe's method of estimating fructose, considerably increasing its accuracy (described in Bacon & Bell, 1948). Cole & Hitchcock (1946) estimated the fructose in the arterial blood of pregnant ewes and in the oxygenated and reduced umbilical bloods of the sheep foetus between the 70th and 144th days. Bacon & Bell (1948), besides providing proof of the existence of both glucose and fructose together in foetal blood, also give estimations between the ages of 107 and 141 days.

None of these workers estimated the two sugars in the amniotic and allantoic fluids nor did they correlate blood sugar values with fluid sugar values. The present work was designed to do this with particular reference to the fructose.

METHODS

Pregnant ewes of known conceptual ages were anaesthetized spinally, using 2–3 c.c. planocaine (procaine) followed by absolute alcohol (1–2 c.c.), preceded by a local injection of procaine (2 c.c.). Cannulae were inserted into the dorsalis pedis artery and vein and delivery was by Caesarean section by the usual technique (Huggett, 1927). The amniotic and allantoic fluids were collected separately. The oxygenated and reduced bloods were obtained with hypodermic needles after formalinizing the umbilical cords to prevent spasm and vasoconstriction (Barcroft, 1946). The total reducing substances were estimated by Hagedorn and Jensen's method and fructose by the original Roe method (1934). (This work was planned and executed before Cole's improved technique was available.)

Here two points should be noted. Partridge (1948) has shown inositol to be present in sheep's foetal blood. This, however, is not estimated by the Hagedorn-Jensen method and so does not rank as a reducing substance determined by this method. Secondly, Bacon & Bell (1948) demonstrated in three sheep embryos that the total reducing substances in foetal blood include not only fructose and glucose but also about 10 mg.% of non-fermentable reducing materials (mainly glutathione and uric acid).

The results reported in this series of sheep therefore include fructose and the non-fructose reducing substances of which perhaps 10 mg.% are non-fermenting and the remainder glucose. No glucose-oxidase or yeast fermentations have been performed.

RESULTS

The results of estimating the fructose in the oxygenated blood of the foetus, the amniotic and the allantoic fluids are shown in Table 1. In the maternal blood of all animals the total amount detectable was too small to be estimated.

Values for the non-fructose reducing substances were obtained by deducting the fructose figures in Table 1 from the total amount of reducing substance present. No subtraction was needed in respect of maternal blood.

It would appear (Bacon & Bell, 1948) that the glucose in the foetal blood is less than the figures in Table 2, by about 10 mg. Neither Bacon & Bell (1948) nor we (our work in this series of sheep was completed before their results were available) have systematically examined the amount of the non-fermentable reducing substances at the different ages of foetal life, and no one has estimated these substances in the amniotic and allantoic fluids.

TABLE 1. Fructose of foetal blood and fluids (mg. %)

Foetal age days	Foetal oxygenated blood	Amniotic fluid	Allantoic fluid
49	—	900	—
65	133	65	375
69	166	80	500
85	67	160	113
100	75	Left 120 Right 87	Left 100 Right —
105	67	200	107
110 MD	83	253	360
115	84	168	—
121	115	300	165
125	80	200	366
130	51	210	60
134	43	87	85
135	38	35	56
140	19	58	51

MD = Maternal parasitic disease of the lungs

TABLE 2. Non-fructose reducing substances (mg. % glucose)

Foetal age days	Maternal arterial blood	Foetal oxygenated blood	Amniotic fluid	Allantoic fluid
49	223	—	55	—
65	70	152	90	380
69	50	29	23	452
85	57	19	1·5	91
100	90	25	Left 120 Right 128	10
105	68	6	31	104
110 MD	257	36	51	7
115	33	0	25	—
121	83	30	25	130
125	53	59	220	28
130	47	19	30	127
134	15	27	39	125
135	44	53	25	160
140	80	81	109	188

MD = Maternal parasitic disease of the lungs.

DISCUSSION

The foetal blood sugars. The blood sugar values for fructose and for the total reducing substances, including fructose, agree reasonably well with those of Cole & Hitchcock (1946) and with those of Bacon & Bell (1948). There is a tendency for sporadically high (greater than 100 mg. %) fructose values in the middle part of pregnancy, between the 60th and 120th days of foetal age, with mean values about 100 mg. %. After the 120th day the values fluctuate widely, but with mean values about 60–70 mg.

The non-fructose reducing materials of the foetal blood shown in Table 2 are probably an index of glucose values, but one would not be warranted in going further than this in view of Bacon & Bell's (1948) findings of a mean value for non-fermentable reducing materials of 6–14 mg. and of glucose (fermentable by glucose-oxidase) of 22 mg.

The sugars of the amniotic and allantoic fluids. The values for fructose and for non-fructose reducing substances are shown in Tables 1 and 2. There is no correlation at any age between the fructose values in the foetal blood and those in the two fluids, and limited correlation between its values in the two fluids at any one age. There is likewise no correlation between the values for non-fructose reducing substances (probably mainly glucose) in the foetal (or maternal) blood and in the two fluids.

Table 1 does show certain very high values for fructose in early foetal life at about the 49th–65th day. There is, however, a more generalized drift to high fructose values between the 100th and 130th day in both fluids, but no evidence that it has any metabolic significance. The figures need confirmation.

The only previous figures for the fluids of the sheep are those of Paton *et al.* (1907) who do not give their method of estimating the reducing sugar. They state that the sugar is fructose, give no consideration to the presence of any other sugar and give tables showing 'Sugar values G' and also 'G/N values'. So far as we know ours are the first recorded values for fructose and non-fructose reducing substances in amniotic and allantoic fluids.

SUMMARY

1. Estimations are recorded of the fructose and non-fructose reducing substances in the foetal and maternal bloods of the sheep and also in the amniotic and allantoic fluids.
2. There was no determinable fructose in the maternal blood.
3. The fructose values tend to exceed the values for non-fructose reducing substances. The latter are probably mainly glucose, but no evidence is advanced on this point.
4. The amniotic and allantoic fluid values for the fructose and non-fructose reducing substances are different from one another. The figures are all higher than the blood sugar values.
5. There is a suggestion of consistent rise of fructose in the amniotic and allantoic fluids from about the 100th to the 130th days of foetal age. After the 130th day the fructose values fall to about 40–50 mg. %.
6. There is no correlation between the concentrations of non-fructose reducing substances in the fluids (amniotic and allantoic) and in the foetal bloods.

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ABSORPTION FROM THE LUNGS

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The protein concentration is high in the fluid coughed up by patients with acute pulmonary oedema (Fishberg, 1940), and in oedema fluid of animals with pulmonary oedema induced by various methods (Cameron, 1948). There is, therefore, considerable evidence to suggest that the resolution of pulmonary oedema, however induced, implies the removal from the lungs of fluid containing a considerable amount of protein.

It is well known that 0·9% NaCl solution is readily and rapidly absorbed from the lungs, but the physiological problems involved in the removal of large amounts of protein-containing fluid from the alveoli have received comparatively little attention. Fox (1936) showed that antibodies were absorbed very slowly from the lungs of rabbits and, reviewing the literature, concluded that proteins in general were not readily absorbed from the lungs. Courtice & Phipps (1946) showed that, in rabbits, water and 0·9% NaCl solution were rapidly absorbed from the lungs, while homologous serum was much more slowly removed. Drinker, Warren & MacLanahan (1937) and Drinker & Hardenbergh (1947) showed that proteins were transferred very slowly from lungs to blood stream in anaesthetized dogs, only the lymphatic pathway being available. From the low concentrations of added foreign protein in right duct lymph of anaesthetized dogs, for periods up to 4 hr. after protein solutions were given intratracheally, Drinker & Hardenbergh (1947) concluded that enzymatic breakdown of protein probably precedes resolution of pulmonary exudates and transudates. They employed positive pressure artificial respiration to accelerate lymphatic flow.

Most experiments on pulmonary absorption have been conducted for relatively short periods on anaesthetized recumbent animals. In the present investigation rabbits have been anaesthetized only during the intratracheal injection, and the subsequent absorption into the blood stream of the blue dye, T 1824, combined with human or rabbit serum proteins studied for periods of 2–6 days, and compared with the absorption of dye from protein-free solutions. To assess the importance of respiratory movement for the removal of protein from the lungs, the absorption of dyed protein has also been determined in a group of rabbits anaesthetized for long periods.

The molar proportion of dye : albumin in the material injected was approximately 8 : 1, viz. 0·4 % dye in 3½ % human serum albumin or in plasma. Rawson (1943) has shown that all the dye combines with serum albumin *in vitro*, in these proportions. As a further test, *in vivo*, protein concentrations have been estimated by precipitin titration and dye concentrations measured after intratracheal and intravenous injection of dye in human serum albumin solution.

METHODS

Rabbits were anaesthetized intravenously with 'Veterinary' Nembutal solution. The trachea was exposed, a narrow bore trochar and cannula introduced and the dye solution injected slowly through the cannula. In all cases 1 c.c./kg. body weight of a 0·4 % solution of dye was introduced. The dye was dissoved in distilled water, saline, or a variety of protein-containing fluids—the rabbit's own undiluted serum or heparinized plasma, 3·5 % pure human serum albumin in distilled water or in saline, human serum or human heparinized plasma. All solutions were prepared freshly on the day of injection. The animal was kept in the supine position with the head and chest raised during the injection and afterwards until recovery from the anaesthetic, which took usually about 1 hr.

Before intratracheal injection and at various periods thereafter for some days blood samples were taken from an ear vein for estimation of plasma dye concentrations and, where applicable, for precipitin titration of human serum albumin. In one set of experiments the amount of dye remaining in the lungs after dye and dye-in-serum albumin injections was also determined at various intervals.

Determination of dye concentrations in plasma. The method of Harington, Pochin & Squire (1940), modified for 1 c.c. samples, was employed. Only one extraction of bile pigments was found necessary. The concentrations of dye in the butyl alcohol extracts were estimated photoelectrically (Courtice & Gunton, 1949).

Determinations of amounts of dye present in lungs. Both lungs were removed with heart attached, after killing the animal by intravenous injection of nembutal, and immediately perfused with 0·9 % NaCl solution to wash out most of the blood. The lungs, with main bronchi attached, were then removed from the trachea and cut up finely. A portion of undyed lung and the rest of the lung tissue were macerated, separately, in a Folley homogenizer and made up with 0·9 % NaCl solution to 30–50 c.c. They were then digested with pepsin and 5N-HCl overnight at 37° C., and, on the following morning, the digests were again triturated and made up to such a final dilution with 0·9 % NaCl solution as would give a suitable dye concentration in the dyed lung portion. One c.c. samples of the diluted digests were extracted, without preliminary washing at pH 3·5, with 5N-HCl and butyl alcohol. Dye concentrations were estimated photoelectrically, calibration curves being constructed with known concentrations of dye in undyed lung digests. The total dye present in the lungs was calculated from the measured dye concentrations and the final volume of digest. In two preliminary experiments 8 and 4 mg. respectively of dye were added to chopped up lungs before using the homogenizer. Using the method described the estimated values for dye were 7·9 and 3·75 mg. respectively.

Estimation of precipitin titres. Whenever human serum albumin solution, human plasma or human serum had been injected intratracheally blood was taken for serum as well as plasma at each bleeding. Serum samples were kept in the refrigerator until precipitin estimations were made. 'Wellcome' rabbit precipitating serum (anti-human) was used throughout. The end-titres of the batches used were approximately the same, and were checked during each set of determinations with known dilutions of the material injected intratracheally. In preliminary titrations the undiluted antiserum was shown to form precipitates with 0·4 % dye in 3·5 % human serum albumin up to dilutions of 1 : 6000 to 1 : 8000. In performing estimations, one drop of test-serum dilution

was layered over one drop of undiluted antiserum in capillary cups on stems. The result was determined after 1 hr. at room temperature. For dilution, 0·9 % NaCl solution dyed with T 1824 was used. In each set of determinations a dyed saline-antiserum control was included and the rabbit's preinjection serum served as a further control. End-points were first determined with successively doubled dilutions; then final dilutions were made up, each increasing by about 30 %, over the end-point range.

RESULTS

To provide a basis for comparison with absorption of dye-protein mixtures, two groups of rabbits were injected intratracheally with the dye in protein-free solution and subsequent plasma-dye concentrations measured. The first group, of twelve rabbits, received dye in distilled water. In some of these rabbits the percentage of dye remaining in the lungs was also determined. The plasma dye concentrations at intervals after administration are shown in Table 1. In all twelve rabbits dye was evidently absorbed rapidly in the first hour after intratracheal injection, and thereafter much more slowly. By 24 hr. plasma-dye concentrations had fallen, usually considerably, and subsequently continued to fall steadily, reaching 1 mg./l. in two rabbits followed for 8–9 days, and 2·8 mg./l. after 6 days in another rabbit.

TABLE 1. Plasma dye concentrations in mg./l. after intratracheal administration to rabbits of dye, 4 mg./kg., in water, 1 c.c./kg. and in 0 9 % NaCl solution 1 c.c./kg.

Hours after injection					
½	1	3	5	24	48
		Dye in water			
11·3	15·3	16·8	18·0	—	—
5·0	6 0	7·0	7·1	—	—
—	6·1	—	9·2	6·1	4·7
—	21·7	—	20·8	12·0	7·0
—	21·0	—	18·1	11·7	—
K 3 days	21·0	—	24·8	13·3	5·8
	19·9		22·4	19·0	10·5
K 2 days	28·0	-	28·2	21·5	12·3
K 1 day	20·1	—	24·5	14·3	—
—	18·0	—	21·8	17·3	12·5
—	14·5	—	16·3	9·3	6·0
K 5 hr.	—	—	30·5	—	—
		Dye in 0·9 % NaCl solution			
	5·4	9·0	9·8	8·1	3·9
	9·8	15·9	18·1	14·3	8·9
	17·2	20·5	22·8	13·0	6·0
	12·8	16·7	19·5	17·0	6·7

Previous investigations (Courtice & Phipps, 1946) have shown that hypotonic solutions are absorbed without signs of lung damage. However, a second group of four rabbits were given dye in 0·9 % saline intratracheally. The subsequent plasma dye concentrations are given in Table 1. From the data in Table 1 and the averaged plasma dye curves in Fig. 1 it is clear that dye dissolved in water passes more rapidly, during the first few hours, from the lungs into the blood stream than does dye dissolved in saline. This difference is presumably due to more rapid initial absorption of water and dissolved dye until electrolyte equilibrium is reached between the fluid in the alveoli and plasma in the capillaries, when capillary absorption will be determined by the colloid osmotic pressure of the plasma less the net hydrostatic pressure. The early appearance of dye in the circulation and rapid increase of dye concentrations to high levels in the first few hours clearly indicate that dye in protein-free solutions is absorbed mainly by way of the pulmonary capillaries.

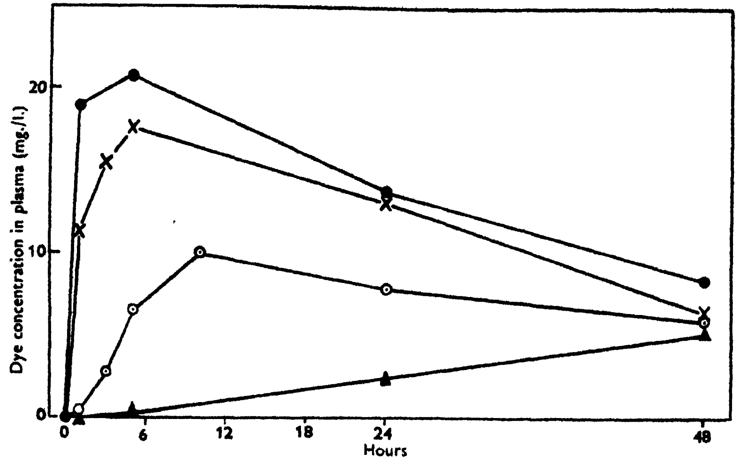

Fig. 1. Average dye concentrations in plasma in mg./l. after intratracheal injection in rabbits of 4 mg./kg. body weight in fluid, 1 c.c./kg. ●, dye in distilled water; ×, dye in 0·9% NaCl solution; ⊙, dye in rabbits own heparinized plasma; ▲, dye in 3·5% solution of human serum albumin.

In studying the absorption of dye combined with serum proteins it was thought advisable to combine the dye first with a pure serum protein foreign to the rabbit. Comparison of changes in precipitin titre with changes in plasma dye concentration would then provide information regarding absorption of protein and dye components of the complex, and thus serve as an indirect test of the stability of the dye-protein complex. Human serum albumin was employed, the source being a commercial preparation (Eli Lilley) of 25 g. human serum albumin in 100 c.c. of buffered diluent, with 1 : 10,000 merthiolate as a preservative. The stock bottle was kept in the refrigerator. Small amounts were withdrawn aseptically immediately before intratracheal injection, made up to 3·5% albumin with distilled water and the requisite amount of dye added. Since diffusion of any uncombined dye into the blood stream should be slow from a solution isosmotic with plasma with regard to both electrolytes and colloids, distilled water was used as a vehicle. It was thought that the presence of substantial amounts of uncombined dye might be revealed by absorption, with water, into the capillaries while electrolyte equilibrium was being established. Drinker & Hardenbergh (1947) have shown that pure bovine serum albumin (crystalline preparation) does not pass into the pulmonary capillaries. The molecular properties of the human serum albumin sample used in this investigation were not investigated in detail. Precipitin titrations of 3·5% albumin solutions gave positive reactions with rabbit anti-human serum up to dilutions of 1 : 6000 to 1 : 8000. Similar end-points were obtained with diluted human serum, showing that the albumin preparation was antigenically effective (in this sense). *In vitro* combination of dye and serum albumin in the proportions used for injection was confirmed by ultracentrifugation (kindly performed by Dr Cecil of the Department of Biochemistry) of a sample of 3·5% albumin plus 0·4% dye in 0·42% NaCl solution. A separating cell was used, and the concentrations of dye and protein in the supernatant were 11 and 125 mg. (w/v)% respectively; that is, 2·5 and 3% of the original dye and protein concentrations. Nearly all the dye sedimented with the protein, and the small percentages remaining in the supernatant could be explained by back diffusion during slowing down of the centrifuge.

After these preliminary investigations dye in albumin solution was administered to a group of seven rabbits. In some of these rabbits the percentage of dye remaining in the lungs was determined. From the individual results in Table 2 and the curve for average plasma dye concentrations in Fig. 1 it can be seen that no appreciable dye appeared in the plasma for the first 5 hr. after dye-albumin. Dye concentrations then rose slowly for the next 48 hr. and, in three rabbits followed for 6 days or longer, reached a peak in 2–3 days and declined slowly. Precipitin titres behaved similarly (Table 2) indicating that dye and albumin were transferred to the circulation together, presumably in combination. To compare the rates of disappearance from the circulation of dye and albumin, 0·2 c.c. of dye-albumin per kg. body weight was injected intravenously into each of two rabbits, and plasma dye concentrations and

TABLE 2. Dye and antigen concentrations in plasma after intratracheal injection in rabbits of dye, 4 mg./kg., in 3·5% human serum albumin 1 c.c./kg.

Plasma dye concentrations in mg./l, first value in each pair, and precipitin titres in serum expressed as reciprocals, second value in each pair, thus no precipitate with undiluted serum = 0, precipitate up to 1:8 dilution = 8.

	5 hr.	1 day	2 days	3 days	5 days
	0, 0	2·2, 2	—	—	—
K 3 days	0·5, 0	1·0, 2	1·7, 4	2·0, 4	—
	0·6, 2	4·5, 16	7·9, 32	—	—
K 2 days	0, –	4·0, 6	5·5, 15	—	—
K 1 day	0, 0	0·5, 0	—	—	—
K 6 days	0, 1	1·8, 2	5·0, 8	3·2, 6	0·9, 4
	0, 0	3·2, 8	5·4, 16	—	—

Antiserum positive up to 1:5000 - 1:8000 dilution of 3½% albumin. In no case did serum taken before intratracheal injection of albumin solution give a precipitate. K 3 days etc., indicates day on which animal was killed to estimate percentage dye remaining in lungs, see text.

serum precipitin titres measured before injection and at 10 min., 5 hr., 1, 2, 4 and 6 days thereafter. The plasma dye concentrations, in mg./l., were 0, 13·7, 8·8, 3·5, 2·0, 0·9, 0·7 and 0, 16·0, 8·5, 5·0, 2·7, 0·9, 0·4 respectively. The corresponding precipitin titres expressed as reciprocals, thus 10 = 1 : 10 highest positive dilution and 0 = negative with undiluted serum, were 0, 60, 40, 20, 20, 10, 5 and 0, 60, 40, 30, 10, 10, 5 respectively, the antiserum giving positive results up to 1 : 6000 dilution of the dye-protein mixture. Thus it can be seen that the disappearance of dye from the circulation runs parallel with that of albumin. Finally, to see whether variations in dye-albumin proportions in the lungs were accompanied by similar variations in precipitin titres relative to plasma dye concentrations, dye-albumin-saline mixtures were injected intratracheally into two rabbits. In both cases 1 c.c. of 0·4 % dye per kg. was injected, but in one case the albumin concentration was 3·5 %, in the other case 14 %. Plasma dye concentrations and precipitin titres were measured before injection and at 1, 3, 5 hr., 1, 2, 3 and 4 days. For the rabbit receiving dye in 3·5 % albumin the results were: Plasma dye concentrations, in mg./l., 0, 0, 0, 0, 2·8, 5·0, 4·2, 2·3. Precipitin titres, in reciprocals, 0, 0, 0, 0, 4, 20, 16, 16. For the rabbit receiving dye in 14 % albumin the results were: plasma dye concentrations, in mg./l., 0, 0, 0, 0·8, 4·0, 5·3, 5·3, 3·1. Precipitin titres, in reciprocals, 0, 0, 2, 8, 64, 160, 128, 128. Thus precipitin titre/plasma dye concentration varied with albumin/dye proportions in the injected material. The high colloid osmotic pressure of the 14 % albumin solution did not increase the delay in absorption.

The technique of precipitin titration employed is not an accurate quantitative procedure but the results obtained demonstrate that plasma dye concentrations and precipitin titres vary together as if the dye and albumin remain combined *in vivo*, and that the absorption of dye-protein is very much slower than the absorption of uncombined dye, with an initial delay of some hours before any absorption is apparent.

It is difficult to estimate from plasma dye concentrations the actual amounts or percentages of dye which have been absorbed from the lungs, since the volume of body fluids in which the dye is distributed and the rate of loss of dye from body fluids are both unknown. Accordingly, some of the rabbits receiving dye in water and dye in albumin solution respectively were killed after predetermined intervals and the amount of dye remaining in the lungs determined as previously described. The rabbits were experimented upon in pairs, one member of the pair receiving dye in water and the other dye in albumin solution. Plasma dye concentrations and precipitin titres have been included in Tables 1 and 2, where the interval after which the rabbit was killed is also indicated thus: K2 days. The percentages of dye remaining in the lungs at various times after intratracheal injection are shown in Fig. 2. This figure demonstrates that there are large quantitative and qualitative differences in the rate of removal of dye and of dye-protein from the lung. Whereas 40% of the dye in water disappeared in 5 hr., none of the dye-protein disappeared in 5 hr. and only 50% had gone by 3 days. After 24 hr. little further dye in water disappeared, whereas dye-protein in the lung continued to decrease up to the third day. Since so few rabbits were studied not too much emphasis should be laid upon this last feature, but the findings are what would be expected from the respective plasma dye concentration curves, and suggest that other channels of loss from the lungs, for example, coughing, contributed but little. No satisfactory method has been evolved for distinguishing quantitatively between the dye remaining in the respiratory tree and the dye in the lung substance in phagocytes, tissue fluid or lymph. It is possibly the dye fixed in the tissues which constitutes the slowly removed residue after dye or dye-protein injections.

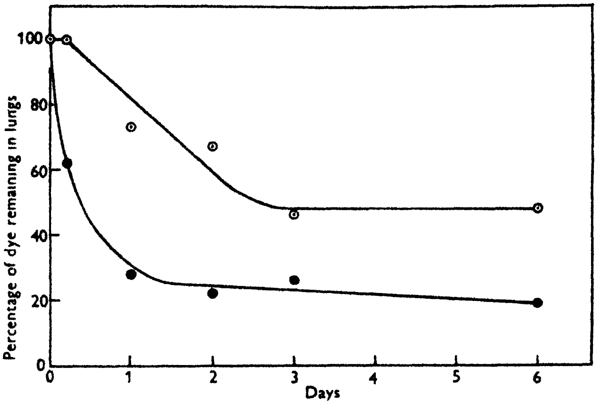

Fig. 2. Percentages of dye remaining in lungs after intratracheal injection in rabbits of dye in water and dye in 3·5% human albumin. Dosages of dye and fluid same as in Fig. 1. ●, dye in distilled water; ⊙, dye in 3·5% solution of human serum albumin.

The composition of the alveolar fluid in pulmonary oedema closely resembles plasma and therefore the absorption of dye-protein from the animal's own heparinized plasma or serum injected intratracheally was investigated. Plasma dye concentrations were measured after injection, the individual results being shown in Table 3 and the average curve in Fig. 1. It is obvious that the dye-plasma protein was much more rapidly absorbed than dye-albumin had been. When dye in human serum or heparinized plasma was injected, Table 3, the dye concentration rose rapidly in the plasma, and precipitin titres showed that antigen concentrations also increased rapidly. The antigenic structure of plasma is, of course, complex, but titres showed that protein must be absorbed *pari passu* with the dye. No explanation can be offered for the slow absorption of the dye-albumin preparation used, but there seems no reason for doubt that the dye-protein from plasma or serum was removed more rapidly in the unanaesthetized animal. Dye-plasma was, however, much more slowly absorbed than dye in aqueous solution and very little dye-plasma was absorbed in the first hour.

TABLE 3. Dye concentrations in plasma in mg./l. after intratracheal administration to rabbits of dye, 4 mg./kg., in plasma or serum, 1 c.c./kg.

Hours after injection					
1	3	5	10	24	48
(1) Rabbit's own heparinized plasma:					
0·3	3·3	10·0	13·3	7·9	5·3
0	3·5	5·4	6·8	5·8	5·4
0	—	0	—	2·1	1·5
0	0	1·2	5·4	7·3	5·7
1·3	4·1	9·5	14·3	14·0	7·3
(2) Rabbit's own serum:					
—	—	7·8	—	13·3	9·8
—	—	0·9	—	11·5	8·4

(3) Human heparinized plasma. Second value in each column = reciprocal of precipitin titre, as in Table 2.

Hours after injection					
1	3	5	9	24	48
0·2, 0	1·4, —	2·8, —	8·0, 64	18·0, 128	8·2, 112
1·1, 4	2·8, —	9·3, —	16·6, 128	18·8, 160	8·5, 128

Antiserum precipitated up to 1:16,000 dilution of human plasma.

(4) Human serum:

1	3	5	9	24	48
0, 0	—	13·6, 48	—	21·0, 96	14·8, 96

Antiserum precipitated up to 1:8000 dilution of human serum. In none of the three rabbits did serum taken before intratracheal injection give a precipitate with antiserum.

The initial delay followed by steady transfer of dye-plasma protein to the circulation suggest that absorption takes place via the lymphatics, but the rate of transfer seems inconsistent with the extremely slow removal of proteins into the right lymph duct of the anaesthetized dog (Drinker & Hardenbergh, 1947). Although species differences might be partly responsible, it was of interest to determine the effect of anaesthesia and recumbency, since both procedures would interfere with respiratory movements and probably with flow in the lung lymphatics.

Dye in their own heparinized plasma was injected intratracheally into five rabbits, in the usual dosage, anaesthesia being maintained for 9 hr. by repeated intraperitoneal injections of nembutal. During this period the rabbits were kept in the supine position, then allowed to recover from the anaesthetic, and further plasma samples were taken at 24 and 48 hr. In Fig. 3 the averaged dye concentration curve for the continuously anaesthetized rabbits is compared with that for rabbits allowed to recover naturally from the initial anaesthetic. It can be seen that anaesthesia plus recumbency markedly decreases the rate of absorption of dye-protein from the lungs, and prolongs beyond 5 hr. the latent period before the first appearance of dye in the circulation. It is unlikely that a similar procedure affects the rate of absorption of uncombined dye, since the plasma dye concentration was 30·5 mg./l. in a rabbit anaesthetized for 5 hr. after injection of dye in water intratracheally. Similarly, in an anaesthetized cat, after injection of dye-saline intratracheally in the same dosage as for rabbits, plasma dye concentrations were 1·4, 3·4, 5·3, 6·7, 7·7, and 8·3 mg./l. at ½, 1, 2, 3, 4, and 5 hr., while dye concentrations in successive hourly specimens of thoracic duct lymph were 0·9, 2·1, 3·5, 5·0 and 5·5 mg./l. the lymph not appearing blue to the naked eye for the first 1½ hr. Thus anaesthesia and recumbency did not delay the absorption of dye-saline nor did breathing 7 % CO_2-air mixture from the third to fifth hour noticeably affect the rate of absorption. On the other hand, in another anaesthetized cat which received dye in its own heparinized plasma intratracheally no dye was visible over a period of 6 hr. in plasma or thoracic duct lymph, although a 7 % CO_2 in air mixture was breathed for the last 4 hr. Dyed lymph was seen in the right lymph duct of the cat which received dye-saline, from about 3½ hr. onward, but in the cat which received dye-plasma no lymphatics containing dye could be found in the neck, and at autopsy dyed lymphatics did not extend further up from the hilus than two lymph glands low down at the right side of the trachea.

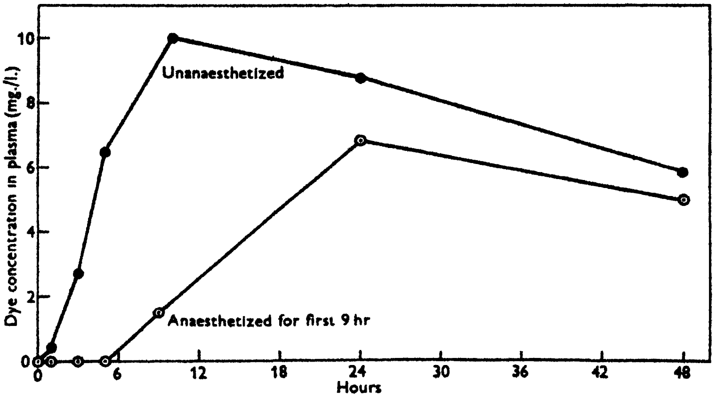

Fig. 3. Average dye concentrations in plasma in mg./l. after intratracheal injection of dye in their own heparinized plasma in rabbits allowed to recover from initial anaesthesia and in rabbits anaesthetized for 9 hr. and then allowed to recover. Dosages of dye and plasma same as in Fig. 1.

DISCUSSION

The site and routes of absorption after intratracheal injection of solutions. Previous investigators have shown that liquids pass rapidly into the terminal divisions of the bronchial tree (Corper & Robin, 1923), and this was confirmed, histologically, during the present investigation. Once in the terminal divisions, the fluid is exposed to a very large vascular and lymphatic absorbing surface, and there seems little doubt that this was where absorption took place after intratracheal injections. It seems unlikely that significant amounts were absorbed from the airway proximal to the respiratory bronchioles, since the absorbing surface is very much smaller and is at a greater distance from the injected material. The absorption rates of dye and dye-protein from the nose and naso-pharynx (Yoffey & Drinker, 1938; Yoffey, Sullivan & Drinker, 1938) are very much slower than those after intratracheal injection, and there are no anatomical grounds for expecting absorption from the rest of the upper respiratory tract to be much more rapid than from the nose. During experiments extending over several days dye coughed up, or passed up the trachea by ciliary action, and subsequently swallowed might be absorbed from the alimentary tract. However, when 1 c.c./kg. body weight of 0·4% dye was injected by oesophageal tube into each of two rabbits no blue dye was seen in the plasma during a subsequent period of 4 days.

Drinker & Hardenbergh (1947) have shown that in dogs serum proteins are absorbed from the lungs by lymphatic channels only. In rabbits, the delay in appearance of dye-protein in the blood stream and the prolongation of this delay by decreased lung movements in anaesthetized, recumbent animals also indicate lymphatic absorption. When dye dissolved in distilled water or in saline is given intratracheally, however, the plasma dye concentration curves clearly indicate absorption directly into the blood stream.

The stability of the dye-protein linkage. Reference has already been made to the investigations of Rawson (1943), who showed that the dye was bound by plasma albumin *in vitro* and that, of four structurally related blue dyes, T 1824 was the least easily removed from combination with protein by cellophane strips and the slowest to disappear from the blood stream in dogs (Gregerson & Rawson, 1943). The experiments on absorption from the lungs of rabbits have provided good indirect evidence for a comparable stability of the dye-protein combination during absorption, viz. the features already discussed indicating lymphatic absorption of dye-protein and vascular absorption of uncombined dye, the absence of any immediate dye absorption after dye-albumin-water intratracheally, the parallel changes in plasma dye and antigen concentrations, the slow removal of dye from the lungs after injection of dye-protein mixtures. The last of these features, considered by itself, could be interpreted merely as a slow diffusion of dye from solutions isosmotic with plasma, but such an explanation would not fit all the facts. Finally, in experiments on absorption of dye-plasma mixtures from the pleural cavities (Courtice & Simmonds, 1949) it has been shown that exclusion of lymphatic pathways abolishes transfer of dye to the circulation, whereas uncombined dye can undoubtedly pass through the capillary wall.

Le Veen & Fishman (1947) have criticized Gregerson and Rawson's evidence for stable dye-protein combination. However, Le Veen & Fishman's demonstration of similar disappearance rates of dye from the circulation in normal and hyproproteinaemic subjects is open to several interpretations, their experiments *in vitro* on equilibrium proportions involved drastic denaturation of the protein, and evidence that an exchange resin dissociates the dye-protein complex requires the further demonstration that comparable forces operate *in vivo*. Fine & Seligman (1943, 1944) showed that radioactive sulphur-plasma proteins from dogs fed with the isotopic amino-acids disappeared more slowly from the circulation of anaesthetized dogs than plasma proteins labelled *in vitro* with radioactive halogens, while the disappearance rates of the blue dye, T 1824, were comparable with those of the iodo-plasma proteins. This suggests that dyed protein is treated in the body differently from undyed protein. The results of an experiment where dyed human serum albumin was injected intratracheally in one rabbit and undyed albumin solution in the same dosage in another are shown in Fig. 4. The similarity of antigen-titre curves shows that there was no gross difference between absorption rates of dyed and of undyed antigen. This finding is not necessarily inconsistent with Fine & Seligman's results. It shows, however, that study of the plasma dye concentrations after intratracheal injection of dyed protein should not lead to a serious overestimation of the rate of absorption of undyed protein.

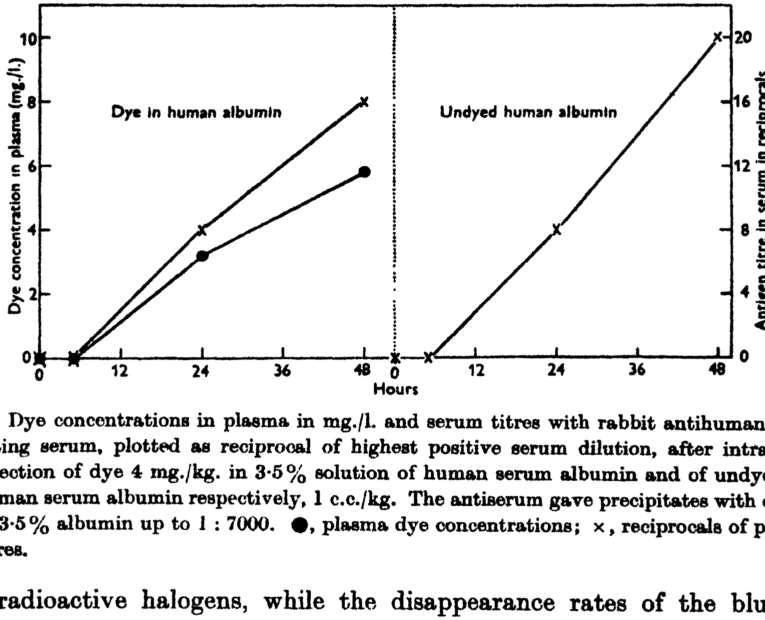

Fig. 4. Dye concentrations in plasma in mg./l. and serum titres with rabbit antihuman precipitating serum, plotted as reciprocal of highest positive serum dilution, after intratracheal injection of dye 4 mg./kg. in 3·5 % solution of human serum albumin and of undyed 3·5 % human serum albumin respectively, 1 c.c./kg. The antiserum gave precipitates with dilutions of 3·5 % albumin up to 1 : 7000. ●, plasma dye concentrations; ×, reciprocals of precipitin titres.

The importance of lung movements in lymphatic removal of proteins from the lung. It has been necessary to discuss in some detail the assumptions involved in using plasma dye concentrations as a measure of dye-protein absorption since plasma dye concentrations indicated quite rapid lymphatic removal of plasma proteins—relative to that in anaesthetized rabbits, in an anaesthetized cat, or in the anaesthetized dogs studied by Drinker & Hardenbergh (1947)—in rabbits moving about freely after recovery from a short period of anaesthesia. From the facts available it cannot be inferred that the dye-albumin linkage is as stable as, say, the bonds between the amino-acids, but the linkage does appear to be sufficiently stable to provide a useful measure of protein absorption. There seems no reason to believe that dye labelling increases the rate of protein absorption and, since A/G ratios in lung lymph are similar to those in lung oedema fluid (Cameron & Courtice, 1946), no reason to believe that albumin is removed more quickly than globulin.

The average plasma dye concentration in the unanaesthetized rabbits at 10 hr. was 10 mg./l. (Fig. 1), and if it is assumed that the plasma volume is approximately 40 c.c./kg. then about 10% of the injected dye protein must have been in the circulation. Since after a single intravenous injection dye concentrations decline by 30–40% in 10 hr. in unanaesthetized rabbits, it is probable that nearly 20% of the dye-protein given intratracheally was absorbed from the lungs in 10 hr.

While, therefore, lymphatic removal of plasma from the lungs is undoubtedly slow compared with the rate of vascular absorption of 0·9% NaCl or water, it appears to be more rapid than would be expected from experiments restricted to anaesthetized recumbent animals. Although species differences cannot be excluded the slow lymphatic removal of proteins in Drinker & Hardenbergh's experiments could be explained on this basis. Slow lymphatic removal despite positive pressure artificial respiration, or expiration against a resistance (Courtice & Phipps, 1946), might be explained by the effect of posture and anaesthesia in restricting mobility of the parts of the lung containing the injected material.

HISTOLOGICAL ADDENDUM

By E. H. Leach

The lungs from similarly treated animals were removed 2 days after intratracheal injection of water, plasma and albumin solution, both dyed and undyed. The material was fixed in Susa and embedded in paraffin. Sections were cut at 20 μ. and counterstained with neutral red; other sections were stained with haematoxylin and eosin.

The injection of the undyed water caused no obvious effect. When dyed water was used, numerous dust cells, containing blue granules, were seen both on the walls of the alveoli and also free within them. Blue-staining coagula were present in the lumen of alveoli and in the connective tissues of their walls.

Both albumin and plasma caused an obvious but variable reaction, consisting of a polymorphonuclear cell infiltration, congestion and perivascular oedema. Coagula were present in alveoli together with numerous dust cells. Coagula were also present in the connective tissue spaces.

Dye albumin and dye plasma caused changes very similar to those described above. No constant differences, attributable to the presence of the dye, could be noted in the extent of the reactions of the tissues. Dust cells containing blue particles were seen in sections of the bronchioles and of the trachea.

SUMMARY

1. Using the blue dye, T 1824, as a labelling agent, the absorption has been investigated of human and rabbit plasma proteins from the lungs of rabbits. The measures of absorption used were the serial plasma dye concentrations, precipitin titres and, in some cases, the percentage of dye remaining in the lungs after various intervals.

2. With human plasma proteins, the similarity in behaviour of precipitin titres and dye concentrations supported the assumption that the dye remains combined with protein during absorption.

3. The absorption of dye-protein is qualitatively and quantitatively different from the absorption of uncombined dye; the dye-protein is absorbed only after an initial delay and at a much slower rate.

4. In anaesthetized recumbent animals, absorption of dye-protein is very slow. None is absorbed in the first 5 hr. and only a very small amount by the end of 9 hr.

5. In animals allowed to recover from anaesthesia after the intratracheal injection, dye-protein is absorbed more rapidly, although still much more slowly than uncombined dye.

6. The evidence that dye-protein, after intratracheal injection, is absorbed from the terminal respiratory tree by lymphatic pathways alone is discussed, together with the bearing of these investigations on the removal of pulmonary oedema fluid.

We should like to thank Dr R. L. Vollum, University Demonstrator in Bacteriology of the Sir William Dunn School of Pathology, for advice on the performance of precipitin titration, and Mr P. J. Phipps for technical assistance. We should also like to thank Mr E. H. Leach, M.A., B.Sc., for advice on histological preparations and for a report on the sections submitted, and Mr Marsland for histological technical work.

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ABSORPTION OF FLUIDS FROM THE PLEURAL CAVITIES OF RABBITS AND CATS

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In acute pulmonary oedema caused by lung irritants such as phosgene, pleural effusion is often observed. The fluid in the pleural cavities has a high protein content and is thought to come from the alveoli. If recovery occurs this fluid should, therefore, be absorbed chiefly by the pleural lymphatics. Little, however, is known of the rate of absorption of fluids from the pleural cavities, since most experimental studies have been concerned with suspended particles.

In 1894, Starling & Tubby, experimenting upon anaesthetized dogs for periods up to 6 hr., showed that the dyestuffs indigo carmine and methylene blue were readily absorbed from the pleural cavities, appearing in the urine before the thoracic duct lymph, and in thoracic duct lymph only in concentrations approximating those in the plasma. They found that pleural fluid composition was so altered as to establish equilibrium with plasma values, as shown by decreased dye colour in residual pleural fluid, or falling glucose or saline concentrations when hypertonic solutions were injected. When hypertonic solutions were used, more fluid was recovered after 2–3 hr. than originally injected. In two out of three experiments with undiluted defibrinated blood, more fluid was recovered than originally injected. In the other experiment 10% of injected fluid was removed in 2 hr. In another experiment 6% of a mixture of equal parts serum and saline was removed in 2 hr. The total fluid injected varied in different experiments from 30 to 50 c.c. in dogs of 4–8 kg. weight.

The results obtained by Starling & Tubby are quoted in some detail since they represent the main source, for the past 50 years, of quantitative information on pleural absorption. Drinker & Yoffey (1941) reviewed the available literature and concluded that lymphatic drainage of the pleural sacs is a slow process, and that one may expect the removal of exudates and transudates to be directly related to their protein concentrations, whole blood disappearing more slowly than diluted serum. They also suggest that, as in the case of the lungs, respiratory movements are of great importance in bringing about lymphatic absorption from the pleural sacs.

Since most of the work done so far has been on anaesthetized animals, for a relatively short time, it was decided to investigate the problem of the rate of removal of fluids from the pleural cavities in unanaesthetized rabbits over a longer period of time, and the pathways of removal in anaesthetized cats.

METHODS

Experiments on rabbits. Under nembutal anaesthesia an intercostal space was exposed on the right side through a skin incision. Muscles were retracted to expose the intercostal muscles over a small area. The material to be introduced was then injected into the pleural cavity from a syringe through a flat bevelled needle. The muscles were replaced, the skin incision sutured and the animal allowed to recover from the anaesthetic, which usually took about 1 hr. Operations were not performed under strict aseptic conditions, but in no case was there evidence of post-operative infection.

The fluid injected was either homologous heparinized plasma or 0·9% NaCl solution in doses of 6 c.c./kg. body weight. In the plasma or saline 4 mg./kg. body weight of the blue dye T1824 was dissolved, in some cases, giving a dye concentration of 0·067%. The dye used was an Eastman-Kodak sample, the same stock bottle as that used in blood-volume determinations by Courtice & Gunton (1949), and in experiments on dye and protein removal from the lungs by Courtice & Simmonds (1949). Plasma for intrapleural injection was usually taken on the previous day from rabbits not used in these experiments, and stored in the refrigerator overnight. Weighed amounts of dye were added on the day of injection when required, and thoroughly dissolved. Saline solutions were made up from freshly distilled water and analar NaCl, dye being added to some samples. Stock solutions of saline or dye-saline were used but no injection was made from stock more than 3 days old. When serum albumin solutions were employed stock solution of 25% pure human serum albumin (Eli Lilley) was added to 0·9% NaCl to give 3·5% albumin concentration. The mixture was made up freshly on the day of injection, when dye was also added.

The actual amount of fluid removed from the pleural cavities was determined by killing the animal after a specified period and measuring the volume of fluid remaining. The rabbits were killed by rapid intravenous injection of nembutal, the chest wall opened without contaminating the pleural contents with blood and the fluid pipetted into a measuring vessel.

Since the blue dye, T1824, combines with plasma albumin (Rawson, 1943), the concentrations of dye in the plasma were determined to give an estimate of the protein absorption. Samples of blood were taken from an ear vein before and at 1, 3, 5, 10, 24 and 48 hr. after intrapleural injection. When human albumin was used, the concentration of this substance in the serum was also estimated by a precipitin test to see whether the dye-protein complex was absorbed as such.

Experiments on cats. These experiments were performed to ascertain the pathway of absorption and the effect of respiratory movements on the rate of absorption. Fully grown cats were anaesthetized with nembutal intraperitoneally, anaesthesia being maintained throughout the experiment by repeated injections when necessary. The right femoral artery was cannulated for blood sampling and a tracheal cannula inserted. The main thoracic duct was cannulated, aberrant vessels being tied off. In some cats the main right lymph duct was successfully cannulated before intrapleural injection or at various intervals thereafter, subsidiary vessels being tied. In other cats right lymph ducts were left intact or tied off at various intervals after intrapleural injection.

The right lymph duct in the dog can often be successfully cannulated (Drinker, 1945; Cameron & Courtice, 1946; Cameron, Courtice & Short, 1947), but in the cat this is sometimes made more difficult, because there are frequently several branches from the chest wall as well as from the lungs entering the vein separately. This is also true to some extent in the dog, but in our experience even more so in the cat. In these experiments, in each of two cats there was one large duct which was cannulated and minor ducts which were ligated; in each of three cats there were a few ducts all approximately the same size, so one was cannulated and the others ligated. Thus some, but not necessarily all, of the lymph which would normally enter the right side was collected.

After preparation of the cat was complete its own heparinized plasma, or 0·9 % NaCl solution in distilled water, was injected into the left pleural cavity in doses of 2 c.c./kg. body weight containing 4 mg. of dye dissolved in each 2 c.c.—that is 4 mg. dye/kg.

Lymph from the thoracic duct and right lymph duct was collected at intervals of ½–1 hr. before and after intrapleural injection for estimation of the volume of lymph and dye concentration. At intervals of 1 hr., 2 c.c. blood samples were taken from the femoral artery for estimation of plasma dye concentrations. When the effects of increased ventilation were being studied, the cat inspired 5–7 % CO_2 in air from large Douglas bags through rubber flap valves attached to the tracheal cannula, and expired to room air. The valves introduced very little resistance to breathing, and the dead space of the system was small relative to the tidal air. Control periods where the cat breathed room air alternated with periods of breathing CO_2 mixtures.

After experimental periods varying from 3 to 8½ hr. the cat was killed by rapid intravenous injection of 2 c.c. nembutal, the trachea tied and the thoracic cavity opened from below. The remaining pleural fluid was measured in some instances, and the thoracic contents invariably carefully inspected. A common post-mortem finding was the presence of air in the superior anterior mediastinal tissues, doubtless due to the opening up of fascial planes during identification and dissection of lymph channels. The apical parietal pleura appeared intact in all cases.

Dye concentrations in lymph and plasma. Determinations were made by the method of Harington, Pochin & Squire (1940), modified for 1 c.c. samples. Plasma samples were washed once at pH 3·5 but for lymph samples preliminary elution of bile pigments was omitted. Dye concentrations in the butyl alcohol extracts were estimated photoelectrically, using calibration curves to relate galvanometer deflexion to dye concentration (Courtice & Gunton, 1949).

Precipitin titres for human serum albumin. In an experiment where dye in human serum albumin solution was injected, dye concentrations and precipitin titres were estimated in portions of the same blood samples as described by Courtice & Simmonds (1949).

RESULTS

Experiments on rabbits. In Table 1 the percentages by volume of saline or plasma absorbed from the pleural cavities after periods of 5, 10, 24 and 48 hr. are shown.

TABLE 1. Percentage volumes of fluid removed from pleural cavities of rabbits

	Saline			Plasma			
	5 hr.	10 hr.	24 hr.	5 hr.	10 hr.	24 hr.	48 hr.
Undyed	12	80	100	29	62	84	—
		44	100	14	24	87	—
		56	—	—	11	—	—
Dyed	15	16	70	20	74	91	100
		28	9*	0·5	53	100	100
		45	77	—	—	100	100
		29	100	—	—	—	—
		13	—	—	—	—	—
Mean	14	39	89	16	45	92	100

Result marked * has not been included in calculating averages.

Where more than 1–2 c.c. of fluid was recovered, fluid was present in both pleural sacs, the amount usually being greater on the side of injection. There are individual variations in the percentage rates of absorption, but on the average there is no gross difference between saline and plasma comparable with the difference observed in experiments on removal of these fluids from the lung.

It can be seen that percentages of volumes of dye-saline absorbed were, if anything, slightly less than those of dye-plasma. These differences cannot be accounted for by an osmotic effect of the dye. They may represent a sampling error due to the small number of animals studied or the dye-saline may have caused some irritation and capillary leakage. The fluid removed after dyed or undyed saline or plasma injections was always turbid, the sediment containing mainly fresh leucocytes and a few red blood cells, and sometimes clotted on standing. Starling & Tubby (1894) noted the presence of coagulable protein in pleural fluid removed after injection of protein-free solutions. It is probable that a small amount of protein-containing fluid normally escapes from the pleural capillaries and that the amount is increased if large volumes of fluid are injected into the pleural sacs.

In Fig. 1 are represented the average plasma dye concentrations over a period of 24 hr. in the rabbits in Table 1, which received dye-saline or dye-plasma intrapleurally. There were large individual variations, but the averaged curves indicate a slower absorption of dye from saline solution than of dyed protein. This decreased concentration of dye in the plasma after injection of dyed saline may be due to the combination of the dye with tissue proteins, whereas with dyed plasma the dye is already combined with the plasma albumin.

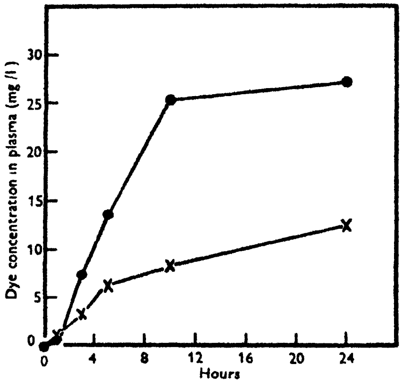

Fig. 1. Dye concentrations in the plasma after intrapleural injection of dye-plasma and dye-saline in unanaesthetized rabbits. ●, dye-plasma; ×, dye-saline.

Table 2. Plasma dye concentrations in mg./l. after intrapleural injection in rabbits of dye-plasma, 4 mg. dye and 6 c.c. plasma/kg.

	Hours after injection*				
	1	3	5	10	24
	(*a*) Unanaesthetized except for first hour				
	0·7	9·0	20·3	35·2	—
	0·7	8·8	16·6	26·0	—
	0·7	6·6	12·5	26·5	31·7
	0·8	11·6	16·8	30·0	28·3
	0·4	3·5	6·0	17·3	24·2
	0·2	4·4	8·6	16·5	24·5
Mean	0·6	7·3	13·5	25·3	27·2
	(*b*) Anaesthetized for 5 hr.				
	0·4	1·3	3·1	—	—
	0·4	1·7	2·4	—	19·2
	0·0	1·4	2·0	—	19·2
Mean	0·3	1·5	2·5	—	19·2

In all these experiments the animals were first anaesthetized but soon recovered, in about 1 hr., and then maintained their normal posture and activity. Since the experiments on cats were all carried out under anaesthesia, a group of three rabbits were injected intrapleurally with dyed plasma and kept anaesthetized for 5 hr., after which one was killed and the other two allowed to recover. Dye concentrations in the plasma were determined at intervals. The individual results for these rabbits and for those which were allowed to recover earlier are given in Table 2. It can be seen that anaesthesia greatly reduces the absorption of the dye-protein.

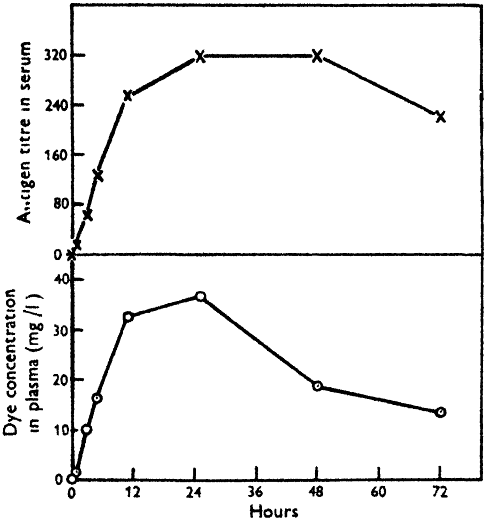

Fig. 2. Dye concentration and antigen titre in the plasma after intrapleural injection of dyed human albumin in the rabbit. ⊙, dye in plasma; ×, antigen titre in serum, as reciprocals.

Absorption of dye in combination with protein. In an experiment where 2 c.c./kg. body weight of 3·5 % human serum albumin in 0·9 % saline, in which dye, 4 mg./kg., was dissolved, was injected into the right pleural cavity, blood samples were taken before injection and at 1, 3, 5, 11, 24, 48 and 72 hr. thereafter. Plasma-dye concentrations and serum-precipitin titres for human serum albumin rose and fell together (Fig. 2), indicating absorption of dye as the dye-protein complex. It thus seems that, as in absorption from the lung, the dye-albumin complex is absorbed as such. If the dye were split off the protein or the protein were broken down by proteolytic enzymes, it is unlikely that the concentration of dye and of human albumin in the plasma would run parallel. This evidence suggests that the rise of the dye level in the plasma gives a good indication of the rate of absorption of plasma albumin from the pleural cavities.

Experiments on cats. At first it was decided to see whether the thoracic duct drained the pleural cavities. In these experiments the thoracic duct was cannulated and the lymph collected, blood samples also being taken at intervals. The cat's own heparinized plasma, 2 c.c./kg. to which 4 mg./kg. dye had been added, was injected into the left pleural cavity. The dye concentrations in the lymph and plasma were taken as a measure of protein absorption. The results of two experiments are shown in Fig. 3. In Fig. 3(*a*) the cat breathed room air throughout, so the pulmonary ventilation and lung movement were small.

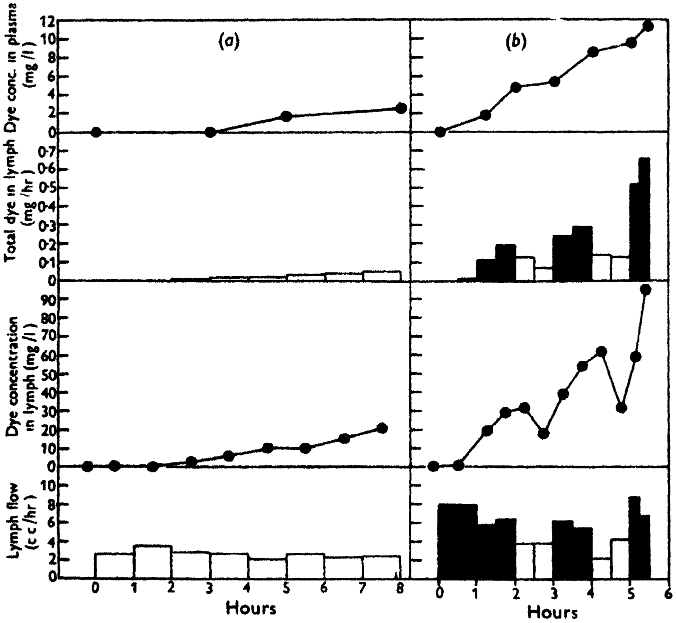

Fig. 3. Lymph flow from thoracic duct and dye concentration in plasma and lymph after intrapleural injection of dyed plasma in the anaesthetized cat. (*a*) Breathing room air throughout; (*b*) breathing 7 % CO_2 in air and room air alternately. Black areas, 7 % CO_2 in air.

Dye did not appear in the lymph until 2½ hr. and then the concentration gradually increased until the eighth hour when the experiment was terminated. There was no dye in the plasma at the end of the third hour, but subsequently the dye gradually increased in concentration. Thus in the anaesthetized cat breathing room air the absorption of dye and presumably of protein is slow, some being absorbed by the thoracic duct, but a greater amount, calculated from the concentration in the plasma and plasma volume, by other routes.

The rate of dye absorption in this experiment was comparable with that in the fully anaesthetized rabbit, but much less than in unanaesthetized rabbits. In order to increase lung movement, experiments were performed in which the cat was allowed to breathe 5 or 7 % CO_2 in air instead of room air. Fig. 3(*b*) shows the results of an experiment similar to that in Fig. 3(*a*) except that the cat breathed 7 % CO_2 in air and room air alternately. The 7 % CO_2 mixture increased the pulmonary ventilation and movement of the chest wall considerably. The effects of this on dye absorption are striking. The lymph flow from the thoracic duct increased with increased lung movement and decreased when room air was breathed. The first appearance of dye in the thoracic duct lymph was much sooner, 55 min. instead of 2½ hr., and the concentration rose rapidly when CO_2 was breathed, falling off again when room air was given. Thus the increased lung movement must have increased the lymph drainage from the pleura relatively more than the diaphragmatic movement increased the lymph flow from the gut. The total amount of dye in the thoracic duct lymph also fluctuated with changes in lung movement.

Although a considerable amount of dye was absorbed by the thoracic duct in this experiment, the dye concentration in the plasma also rose considerably, the rise being affected by lung movement. It can be seen that there is a steep rise while 7 % CO_2 in air is being administered and only a small rise when room air is breathed. Thus lung movement must considerably affect the absorption of the dye-protein complex by channels other than the thoracic duct. In this experiment the cat weighed 3·8 kg. so 15·2 mg. dye was injected. In the 5½ hr. 0·95 mg. or 6·2 % of the dye was collected in the thoracic duct lymph. Assuming that the plasma volume of the cat is 40 c.c./kg., the amount of dye in the plasma at the end of the experiment was 1·76 mg. or 11·6 % of the injected dye. As some of the dye in the plasma would leak out into the tissue fluid after absorption, the rate of removal by other channels must have been even greater than this.

Post-mortem examination of the rabbit and of these cats pointed to the right lymph duct as a channel of absorption, since this duct was deeply blue at the end of experiments. It was, therefore, decided to attempt to cannulate the right lymph duct as well as the thoracic duct in cats. Fig. 4 depicts the results of an experiment on a cat in which the thoracic duct was first cannulated and lymph collected for 5½ hr. Up to this time the results are similar to those in Fig. 3(*b*). The thoracic duct lymph flow, the concentration of dye in this lymph and the dye concentration in the plasma were all affected by lung movement as previously. At 5½ hr. the right lymph duct, which by now was very deeply blue, was cannulated. Fortunately in this experiment, there was on the right side one large duct, the one cannulated, and only a few small lymph channels which were ligated. Then for a further 3 hr. the experiment was continued, 2 hr. with the cat breathing 7 % CO_2 in air and 1 hr. room air.

The results are again quite striking. The right lymph duct flow was greater with increased lung movement, the concentration of dye was very much greater than in the thoracic duct lymph, and the total dye removed by the right side was also much greater than by the left. The cat weighed 2·8 kg. so 11·2 mg. of dye was injected. During this last 3 hr., 0·31 mg. was collected in the thoracic duct and 1·32 mg. in the right lymph duct. The other outstanding feature is that before the right lymph duct was cannulated the dye concentration in the plasma rose fairly rapidly especially when 7 % CO_2 in air was being breathed, but when the lymph from the right side was being collected, the plasma concentration remained steady. This indicates that all, or practically all, the dye being absorbed was now being collected in the thoracic duct and right lymph duct lymph. It seems evident, therefore, that in the pleural cavities the dye-protein complex is absorbed entirely by these lymphatics.

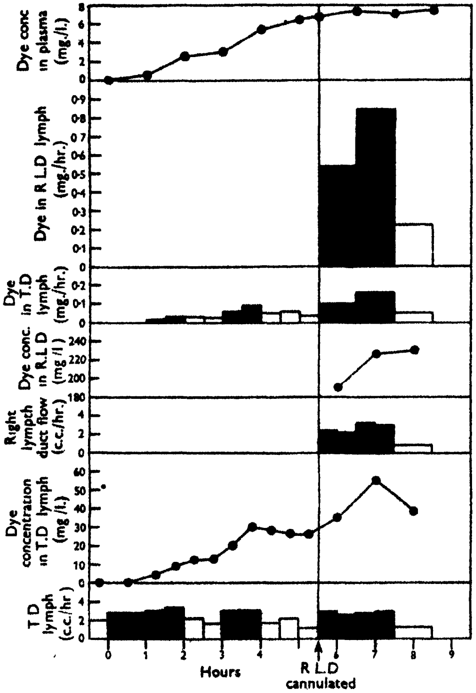

Fig. 4. Lymph flow from thoracic duct and right lymph duct after intrapleural injection of dyed plasma in the anaesthetized cat. Black areas, breathing 7 % CO_2 in air; white areas, breathing

In a similar experiment, the thoracic duct was cannulated as before, and 1½ hr. after the intrapleural injection of dyed plasma, the right lymph duct was cannulated. The experiment was continued for a further 4 hr. with the cat breathing 7 % CO_2 in air and room air alternately. The general picture was similar to that shown in Fig. 4. The cat weighed 5·2 kg., so 20·8 mg. dye was injected. In the period of 4 hr. when both ducts were cannulated, 0·37 mg. dye was collected from the thoracic duct and 1·05 mg. from the right lymph duct.

The plasma dye concentration, which rose slightly in the first 1½ hr. before cannulation of the right lymph duct, remained steady during the 4 hr. when lymph was collected from both ducts.

In three further experiments, both thoracic duct and right lymph duct were cannulated before the dyed plasma was injected into the pleura. In these experiments there were several lymph vessels entering the vein on the right side, so the largest was cannulated and the others ligated. It is possible that one or two small vessels were missed and not ligated. A typical experiment is depicted in Fig. 5. It is evident that in 3 hr. there is but a small amount of dye in the plasma. This may have entered by tiny lymph vessels not ligated. In all three experiments, visible dye appeared in the right lymph duct in 10–15 min. and in the thoracic duct in 50–55 min. with the animals breathing 7 % CO$_2$ for the first 2 hr. The dye concentration in the right lymph duct lymph was much greater than in the thoracic duct lymph, but the total amounts of dye were much the same from both.

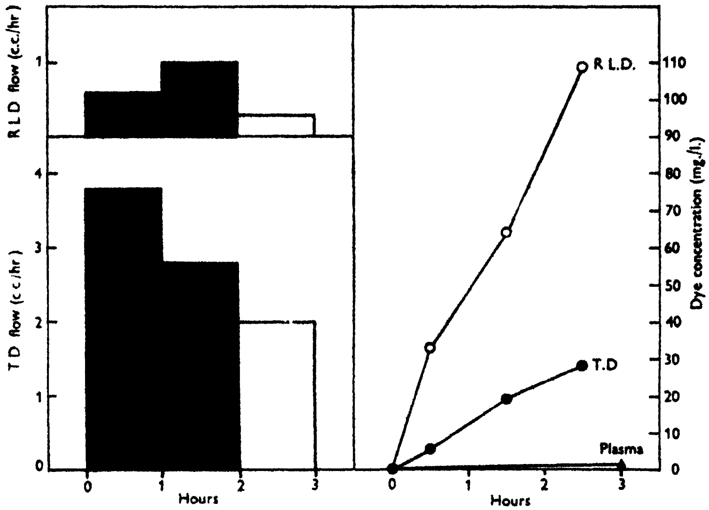

Fig. 5. Lymph flow from thoracic duct and right lymph duct after intrapleural injection of dyed plasma in the anaesthetized cat. Black areas, breathing 7 % CO$_2$ in air; white areas, breathing room air.

Post-mortem examination of all cats and rabbits showed no dye in the abdominal cavity. Even with the animal supine and the head higher than the abdomen, the dye was rapidly driven into the mediastinal tissues and then onwards to the apex of the thorax. From naked eye appearance there was no obvious absorption through the lung tissue—this remained undyed. There appears, therefore, to be little connexion between the pleural and lung lymphatics.

DISCUSSION

It is of some interest to compare the rates of absorption of fluid and of dye found in these experiments with those found under similar circumstances for lungs of rabbits by Courtice & Phipps (1946), and Courtice & Simmonds (1949).

Rate of absorption of fluid. Using comparable doses (10–15 c.c. per rabbit) and assessing the amount of fluid remaining by changes in lung/heart weight ratios, Courtice & Phipps found that homologous serum was absorbed very slowly from the lungs. There were very large individual variations but in most cases it appeared that the serum was not completely removed until periods of 3 days or more had elapsed. Also after the acute phase in phosgene poisoning it takes several days for the oedema fluid to be cleared. In the present investigation, employing doses of 6 c.c./kg. body weight homologous heparinized plasma intrapleurally (10–20 c.c. per rabbit), most of the plasma was absorbed from the pleural cavities by the end of 24 hr. (Table 1).

Similarly Courtice & Phipps found that 0·9% NaCl was absorbed at first very rapidly from the lungs, then more slowly; the initial rate of absorption of water was more rapid than that of 0·9% NaCl. In the present investigation, although smaller numbers of animals have had to be used, the collected data (Fig. 1) suggest that the initial rate of pleural absorption of saline is less rapid, but that by the end of 24 hr. most of the saline is absorbed.

To understand these differences it is necessary to compare the features of the absorption mechanism in lungs and pleura. When a given dosage of fluid is introduced into lungs and pleural cavities respectively, the vascular surface available per unit volume of fluid to be absorbed is greater in the case of the lungs. The balance of hydrostatic pressures will be different in the two situations, although the visceral pleural layer is supplied by the pulmonary artery in animals with thin pleural membranes, such as cat, dog, rabbit (Miller, 1937). Finally, when an originally protein-free solution is introduced it will, if it remains long enough, come to contain significant concentrations of protein, the rate of protein exudation being determined by normal leakage from capillaries and experimental manœuvres such as interference with oxygen supply to alveolar capillary walls by fluid in the alveoli (Drinker, 1945).

There are also differences in the arrangement of the lymphatic apparatus which may be of significance in the absorption of protein-rich fluids. In the lungs, the lymphatic capillaries do not extend farther distally than the end of the alveolar ducts (Miller, 1937), and material in alveoli and atria is separated by some distance from the lymphatic absorbing surface. The significance of this is, however, difficult to assess. There are two routes which may finally be taken by the lymph from the pleural sacs, the thoracic duct and the right lymph duct, while the majority of the lymph from the lungs in the dog and probably in other mammals passes by way of the right lymph duct alone (Warren & Drinker, 1942).

It seems that the absorption of saline from the lungs of rabbits is at first more rapid than from the pleural sacs because a larger absorbing surface is presented, and because the balance of forces over the whole absorbing surface is more favourable in the lungs. After a certain period has elapsed the originally protein-free fluid has become enriched with protein and absorption depends on the removal of protein.

The removal of protein-rich fluids is more rapidly effected from the pleural cavities. Perhaps the availability of both thoracic duct and right lymph duct drainage and the closer approximation of lymphatic absorbing surface may be significant advantages in pleural sac drainage.

The values obtained for absorption of protein-free fluids from the pleural sacs of rabbits are of the same order as those obtained by Starling & Tubby, using dyed solutions in anaesthetized dogs, but slower than in some of their experiments with saline and water. Karsner & Swanbeck (1920), investigating the absorption of carmine and lampblack suspensions from the pleural cavities of cats, found that all the fluid (15 c.c.) was absorbed in a cat killed at 26 hr. but not in four cats killed at 1, 4, 6, and 12 hr. respectively.

The values obtained for absorption rates of homologous heparinized plasma in our unanaesthetized rabbits were much greater than those found by Starling & Tubby for defibrinated blood in anaesthetized dogs. These differences will be discussed when the effect of respiratory movements is considered.

At least four points of difference enjoin caution in extending results on pleural absorption in experimental animals to similar situations in man. In experimental animals the pleural investment is thin, the visceral layer is supplied by the pulmonary artery, the mediastinum is mobile (Berck, 1932) and material injected into one pleural cavity is almost invariably recovered later from both sides.

Rates of absorption of dye. None of the individual dye-saline curves reveals an initial steep rise of dye concentration comparable to that obtained after protein-free dyed fluids in the lungs, where the rise of plasma dye concentration was obviously falling off rapidly by the end of 5 hr. There is, therefore, an initially increased rate of dye absorption from lungs but not from pleural cavities, comparable with the difference found when the rates of absorption of fluid are measured.

The plasma dye curves after introduction of dyed plasma into lungs and into pleural cavity are of the same general form, viz. an initial lag period of 1 hr. or so, then a steady rise to peak values of dye concentration presumably between 10 and 24 hr. The peak values have been missed but the curves suggest they were higher after intrapleural than after intratracheal injection. Dye in plasma is almost certainly transferred to the blood stream in combination with the protein. The curves, therefore, suggest a more rapid absorption of protein from the pleural sacs, comparable with the more rapid absorption of plasma as a whole. Some doubt must always remain in interpretation of dye curves, due to unmeasured loss of dye from the circulation and fixation of the dye in the tissues.

Channels of absorption

The experiments on anaesthetized cats with cannulation or ligation of lymphatics provide good evidence that when dyed protein solutions were introduced into the pleural cavities all the dye that was subsequently removed passed by way of the lymphatics. The experiment in the present series with dye in human serum albumin solution agreed with the experiments on lung absorption in showing parallel changes in dye concentration and antigen titre.

It is difficult to assess the relative importance of the two channels finally available for absorbed material—thoracic duct and right lymph duct. The presence of chyle in the right lymph duct of two of the cats showed that anastomotic connexions were functioning in some cases. Undoubtedly dyed protein was being absorbed from both visceral and parietal pleural layers—on one occasion a small tributary from the anterior superior chest wall was isolated on the left side, and was seen to fill with dye at about the same time as the right lymph duct and to darken in much the same fashion. Despite smaller lymph flows the right lymph duct usually carried away more dye per hour than the thoracic duct. Post-mortem the mediastinal tissues were the most deeply dyed in both the anaesthetized cats and the unanaesthetized rabbits moving freely about the laboratory, but the deepest stained tissues are not necessarily the most active absorbing sites. In the anaesthetized cats the sites of greater absorption will be determined by gravitation of the fluid, as Karsner & Swanbeck (1920) showed for absorption of particulate suspensions.

Effect of respiratory movements

The experimental results presented on lymphatic removal of dye-protein complex, in cats alternately breathing CO_2 mixtures and room air, provide a striking demonstration of the effectiveness of respiratory movements in promoting lymphatic drainage of the pleural sacs. The sustained rise of dye concentration in the lymph with greater ventilation shows that a real increase of lymphatic absorption occurred, not merely a flushing out of dye-filled capillaries by increased formation of lymph elsewhere. There was no evidence that the artificial pleural effusion increased the rate of lymph flow. The steady increase of dye concentration in the lymph with the passage of time is interpreted as evidence of a steady transfer of dyed protein to the lymphatic capillaries, where it mixes with the undyed lymph already there and is moved onwards to the lymphatic trunks by respiratory movements. With increased respiratory movements the onward movement of the mixed lymph is accelerated, the dye concentration increases in the partly emptied lymph capillaries and the dye concentration in the mixed lymph will therefore increase more rapidly. On return to quiet breathing the dye concentration will fall—after a lag period—where a large volume of lymph from other sources is entering the common trunk (viz. thoracic duct). Where the rate of lymph formation from other sources is small the dye concentration in the common trunk will not decrease to the same extent or may continue to rise (e.g. right lymphatic duct).

No measurements were made of the increase of ventilation produced by the 5–7 % CO_2 mixtures. The breathing was obviously greatly increased and the excursions of the chest wall appeared to be increased more than the diaphragmatic movements. Respiratory rates remained steady or increased by 1–2 per minute on the CO_2 mixtures.

The effect of respiratory movements on lymphatic removal probably explains the discrepancy between the slow rates of removal of proteinized fluid in anaesthetized dogs (Starling & Tubby, 1894), and the relatively rapid rates with comparable doses in the rabbits in this series. The dosage of fluid employed in the cats in this series was much smaller and the fluid removed was not measured in all cases. In three cats 19, 37 and 63 % of the plasma injected was removed in 5½, 5½ and 8½ hr. respectively, and 9, 23 and 24 % respectively of the dye injected had been recovered in the lymph or was present in the blood at the end of the experiment. The dye unaccounted for was probably present in the deeply stained mediastinal tissues or had been lost from the blood in the course of the experiments. In these three anaesthetized cats, with ventilation enhanced by CO_2 mixtures during part of the experiment, the rate of removal of plasma is comparable with that for larger doses in unanaesthetized rabbits.

SUMMARY

1. The absorption rate of 0·9 % NaCl and of plasma injected into the pleural cavities of normal rabbits has been investigated.

2. Anaesthesia slows down the absorption, but in unanaesthetized animals both saline and plasma are absorbed at about the same rate. When 6 c.c./kg. of fluid is injected, practically all is absorbed in 24 hr. in contrast to the much slower absorption of plasma from the alveoli.

3. The routes of absorption have been studied in the anaesthetized cat using plasma labelled with T 1824. Evidence suggests that the dye-protein complex is absorbed as such. Absorption occurs only by way of the lymphatics, much more passing through the right lymph duct than through the thoracic duct.

4. The rate of absorption is greatly increased with increased lung movement.

We should like to thank Mr P. J. Phipps for technical assistance.

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HEALING OF ARTIFICIAL DEFECTS IN EMBRYONIC BONE GRAFTS

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The following account is based upon experiments which were originally undertaken in connexion with the hypothetical substance believed to induce ectopic osteogenesis (Bertelsen, 1944; Lacroix, 1945; Levander, 1940, 1945; Levander & Willstaedt, 1946) or to stimulate the repair of fractures (Blum, 1944). The object was to test the ability of various tissue extracts to accelerate the healing of standard artificial defects created in standard bone fragments living under direct microscopical observation. Technical difficulties proved to be such that this intention had to be abandoned, but some interesting observations of a general nature were made and are now reported.

METHODS

Single fragments of chick embryo bone were grafted to the developing chorioallantoic membrane of other embryos, observed, and photographed daily, by the technique described previously (Hancox, 1948). The fragments used were taken always from the same site of the different donor bones to ensure as far as possible a constant trabecular arrangement; before grafting, a standard circular hole was punched out of the fragments with a sterile sharpened stainless steel tube. Some of the preparations were fixed in neutralized formol saline, excised from the membrane, stained by the von Kossa method and mounted whole without sectioning.

RESULTS

In favourable preparations, the central defect healed with unexpected rapidity. After 3 days had elapsed, quite obvious trabeculae had appeared (Pl. 1, figs. 1–4), and by the fifth to seventh day the defects were almost healed (Pl. 1, figs. 5, 6). After 8 days it was often impossible to discern the site of the defect. In many cases it was clear that the developing vascular pattern foreshadowed the future disposition of the trabeculae; for instance, in fig. 2 capillaries can be seen at the second day running radially inward from the grafted bone, over the edge of the defect, toward the centre. Next day, bone trabeculae are present in this site and other radially disposed trabeculae present have clearly arisen in association with other similarly orientated capillaries.

DISCUSSION

Two points of interest arise. In the first place, the extensive formation of bone leading to functional repair of the defects took place in spite of the lack of a nerve supply (the chorioallantoic membrane is devoid of nerves). Niven (1931) demonstrated the repair, following incision with a sharp knife, of mouse and chick embryo long bones isolated in tissue culture, although the amount of bone laid down in her experiments seems to have been considerably less. Corbin & Hinsey (1939) found no changes in the denervated bones and joints of cats even after 3 years. On the other hand, it has been suggested that the nervous system may play an important role in the tissue reactions which bring about fracture healing (Hurrell, 1939). Lower in the evolutionary scale, at least, it is known that regeneration is contingent upon an intact nerve supply (Needham, 1942).

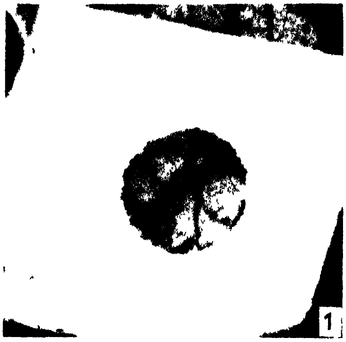
Furthermore, Butler & Schotte (1941) have shown that, far from regeneration, a massive *degeneration* of forearm tissue—including skeletal tissues—occurs after transection of the limbs of urodele larvae previously denervated by repeated division of the root of the brachial plexus of the ipsilateral side.

The present experiments suggest that at least in flat bones such as those of the embryo skull there is an inherent ability to heal defects independently of the nervous system. Nevertheless, the mechanism is relatively simple, consisting in all probability of the migration and proliferation of osteogenic cells, their orientation perhaps in respect to capillaries, and the laying down of fresh trabeculae. It may well be that the more complex changes such as cartilage formation and endochondral ossification, included in the process of repair of long-bone fractures, are dependent upon and controlled by the nervous system. The experiments also afford additional support for the view previously put forward that the trabecular disposition is determined by the developing vascular pattern (Hancox, 1948).

SUMMARY

1. Repair of artificial defects takes place in bone fragments grafted to the developing chick chorioallantois, in spite of the absence of a nerve supply.

2. It is considered that the developing vascular pattern determines the disposition of the newly deposited bone trabeculae.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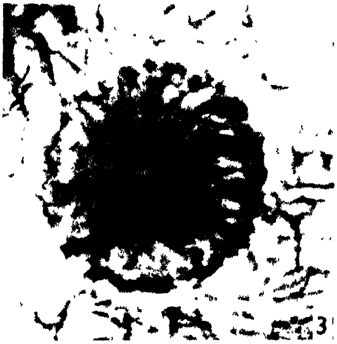
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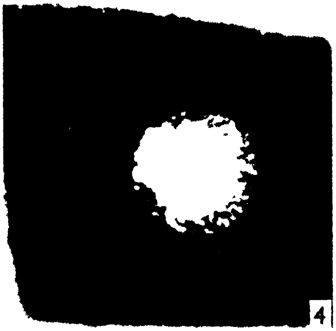
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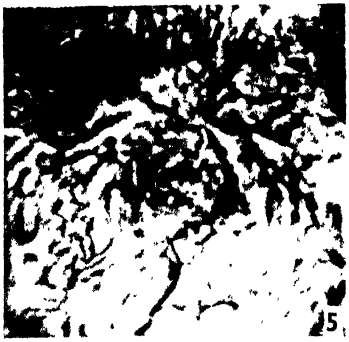
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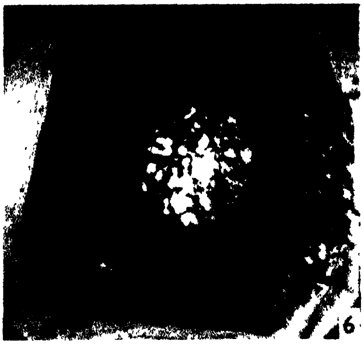
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Figs. 1–6.

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EXPLANATION OF PLATE

Fig. 1. Egg 140. Fragment at start of incubation. Vessels of the host membrane can be seen through the central artificial defect in the bone fragment. (× 60.)

Fig. 2. Same, 48 hr. later. Newly developed capillaries are present and can be seen at the top of the fragment near the edge of the defect, and in the connective tissue within the latter, in the lower right-hand quadrant. (× 115.)

Fig. 3. Same, 24 hr. later. Bone trabeculae are now present, running radially inward from the edge of the defect. (× 115.)

Fig. 4. Same, after fixation and staining with von Kossa technique, showing calcified trabeculae. (× 60.)

Fig. 5. Egg 147. Fragment after 6 days' incubation. The outline of the hole can scarcely be distinguished. (× 115.)

Fig. 6. Same, after fixation and staining. (× 60.)

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THE SIMULTANEOUS ESTIMATION OF THE ABSORPTION OF WATER AND SULPHAGUANIDINE FROM THE STOMACH OF MAN

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The absorption of water from the stomach of man has been established by the observations of Penner, Hollander & Post (1940). However, no estimates of the volume absorbed appear to have been made in man, although Sleeth & Van Liere (1937) have made measurements in anaesthetized cats. These authors showed that a mean of about 6·5% of 200 ml. of water instilled into the stomach was absorbed in 3 hr. Since the rate of absorption was not influenced by the death of the animal, the process is presumably passive. There is support for this view in the work of Bandes, Hollander & Glickstein (1940) who showed that water was not absorbed when the gastric contents of dogs were isosmotic with plasma.

This paper describes experiments in which the absorption of water from the stomach of man has been estimated simultaneously with the absorption of sulphaguanidine. This substance has been shown (Hunt, 1947) only to be absorbed when the osmotic pressure of the gastric contents is less than that of plasma. Thus, since the absorption of water and sulphaguanidine are both influenced by osmotic pressure, it appeared possible that their absorption might be related. If this were so an estimate of the absorption of water might perhaps be made from a measurement of the absorption of sulphaguanidine which is technically the simpler measurement to make.

THEORETICAL CONSIDERATIONS

The plan of the experiment. If fluid is introduced into a stomach, and subsequently recovered, the following simple relationship holds:

$$V_{\text{rec.}} = V_{\text{in.}} + V_{\text{sec.}} - V_{\text{pyl.}} - V_{\text{abs.}}, \tag{I}$$

where $V_{\text{rec.}}$ = volume recovered, $V_{\text{in.}}$ = volume ingested, $V_{\text{sec.}}$ = volume secreted, $V_{\text{pyl.}}$ = volume passing pylorus and $V_{\text{abs.}}$ = volume absorbed. The volume recovered and the volume ingested are known values. To find the volume absorbed, it is necessary to estimate the volume of secretion and the volume passing through the pylorus.

The calculation of the volume of gastric secretion from the amounts of Cl^- *and acid secreted.* It appeared possible that the volume of gastric secretion might be calculated from the amounts of Cl^- and acid secreted by the stomach. Ihre (1938) made a detailed study of the gastric secretion of man in twenty-four normal subjects, stimulating secretion first with histamine and then with insulin. In his results he gave the concentrations of Cl^- and acid and the volume secreted/hr. for each of these stimuli. Using Ihre's values the amounts, expressed in milli-equivalents, of Cl^- and acid associated with each volume of juice have been calculated. The difference between the amount of Cl^- and acid contained in each measured volume of juice has been found, and this has been taken as the amount of so-called neutral Cl^-, i.e. Cl^- not in electrical equilibrium with hydrogen ions.

From these values a regression plane of volume of gastric juice (dependent variable) on amounts of acid and neutral chloride has been calculated by the method of least squares to pass through the origin. This gives an equation

$$V = 5{\cdot}798H + 7{\cdot}448N, \tag{II}$$

where V = vol. of juice in ml., H = amount of acid in m.equiv., N = amount of neutral chloride in m.equiv. The mean difference between the volume of juice *actually recovered* by Ihre from the stomach and the volume *calculated* from the amounts of acid and Cl^- recovered is 3·15 ml. irrespective of sign, with a standard error of an individual difference ± 2·54 ml. Equation II has been used to calculate the volume of gastric secretion responsible for the amounts of Cl^- and acid secreted by the stomach during the experiments reported in this paper.

The calculation of the volume of gastric contents passing through the pylorus. Since the volume passing through the pylorus could not be measured directly, it was calculated in the following way. Phenol red was incorporated in the test solution ingested. This dye has been shown by Penner, Hollander & Saltzman (1938), Shay, Gershon-Cohen & Fels (1939) and Hunt (1947) not to be absorbed, adsorbed or secreted by the gastric mucosa. The difference between the amount of phenol red ingested and the amount later recovered from the stomach was taken to be the amount of phenol red which had passed through the pylorus. Assuming a reasonably steady rate of change in the concentration of phenol red in the gastric contents due to dilution by secretion, the mean concentration of the phenol red in the gastric contents was taken to be half the sum of the concentrations at the beginning and at the end of the experiment. The amount of phenol red lost through the pylorus divided by the mean concentration at which it was lost gave a volume which was taken as the volume of gastric contents passing through the pylorus during the experimental period. Using this volume, and the mean concentrations of chloride and acid, allowances have been made for the amounts of these constituents passing out of the stomach through the pylorus.

Practical application of equation I. Let us reconsider the original simple relationship

$$V_{\text{rec.}} = V_{\text{in.}} + V_{\text{sec.}} - V_{\text{pyl.}} - V_{\text{abs.}}. \tag{I}$$

For anatomical reasons it is difficult to empty the stomach completely. Thus from a practical standpoint equation I should be rewritten:

$$V_{\text{rec.}} = V_{\text{in.}} + V_{\text{sec.}} + V_{\text{rem.}_1} - V_{\text{pyl.}} - V_{\text{abs.}} - V_{\text{rem.}_2}, \tag{III}$$

where $V_{\text{rem.}_1}$ = volume remaining in the stomach immediately before the ingestion of the test solution, $V_{\text{rem.}_2}$ = volume remaining in the stomach after aspirating the gastric contents. $V_{\text{rem.}_1}$ and $V_{\text{rem.}_2}$ cannot be measured directly. but allowances can be made for them in the following way.

$V_{\text{rem.}_1}$. This fluid would consist of so-called 'resting juice', a mixture of saliva, gastric secretion and possibly bile, and would represent an error of unknown and varying degree in the ingested meal. To eliminate this variable error, the stomach has been washed out before each meal with saline of a concentration equivalent to that of neutral gastric juice. This procedure permits of the calculation of $(V_{\text{sec.}} + V_{\text{rem.}_1})$, without their separate evaluation, from the amounts of Cl^- and acid recovered.

The strength of NaCl solution required for this preliminary washout can be determined from the following consideration of equation II:

$$V = 5{\cdot}798H + 7{\cdot}448N. \tag{II}$$

If $H = 0$, i.e. the gastric juice is neutral,

$$V = 7{\cdot}448N.$$

If $V = 1$ ml. $N = 0{\cdot}134$ m.equiv. neutral Cl^-.

Thus, if each ml. of $V_{\text{rem.}_1}$ contains 0·134 m.equiv. NaCl, the use of equation II will ensure that any value of $(V_{\text{sec.}} + V_{\text{rem.}_1})$ will be correctly computed from the amounts of Cl^- and acid recovered plus the amounts calculated to have been lost through the pylorus. In other words, equation II is true not only of gastric juice but also of mixtures of gastric juice and a saline containing 134 m.equiv. Cl^-/l.

$V_{\text{rem.}_2}$. After withdrawing the gastric contents, the stomach is immediately washed out with water, the phenol red content of this wash determined and its equivalent volume of gastric contents calculated.

In this way the difficulty of emptying the stomach completely before and after the experiment began has been overcome. It is thus possible to estimate $V_{\text{abs.}}$ since it is the only unknown in equation III.

In order that the volume available for absorption from the stomach should be kept as large as possible, use has been made of the effect of the intra-duodenal instillation of olive oil in delaying gastric emptying (Shay *et al.* 1939).

METHODS

The following technique has been used to intubate the duodenum. The subject was given 2 m. of stiff rubber tubing 3 mm. in external diameter, and having several perforations a few cm. from one end. He swallowed about 1 m. of tube before his evening meal and a further 50 cm. during the subsequent hours. During the night the upper end was pinned on to his pyjamas whilst the lower perforated end was swept through the pylorus with his supper. On the following morning he came to the laboratory without breakfast and swallowed a soft rubber tube, 2 mm. external diameter. The subject sat in a chair, continuous aspiration of saliva from the mouth was begun and about 30 drops/min. of olive oil were then run continuously into the stiff rubber tube which was withdrawn until it was thought to be in the duodenum. After 10 min. the subject drank 400 ml. of a solution of NaCl (130 m.equiv./l.). (A later calculation gave 134 m.equiv./l.: the error is considered to be insignificant in this context.) As much as possible of this solution was drawn out through the soft tube at once. If no oil was recovered with this saline, 500–800 ml. of a solution containing about 100 mg. of phenol red/l. and about 100 mg. sulphaguanidine/l. at a pH 6·5 were run into the stomach through the soft tube during a period of 5–8 min. This solution has a depression of freezing-point of about 0·05. In five experiments 0·5 mg. histamine acid phosphate was then injected subcutaneously. One hour later the gastric contents, which were never contaminated with oil, were recovered through the soft rubber tube and their volume measured. The subject then drank 200 ml. of water which were recovered through the soft tube and the volume measured. This washout was then repeated. The concentrations of sulphaguanidine and phenol red in the original test solution and in the recovered gastric contents and washout solutions were determined by methods previously described (Hunt, 1947). The concentration of acid was determined in the sample first recovered by titrating to a pH of 6·5 determined electro-potentiometrically. Since the isoelectric point of one group of the buffers present in gastric juice is pH 6·5 (Hori, 1933) the titration is as far as possible independent of the concentrations of these substances if it is assumed that they are secreted at their isoelectric point. In any case the probable magnitude of the error from this source is small. The concentration of Cl^- ions was then determined by titrating with 0·5N-silver nitrate in the presence of small amounts of nitric acid until the e.m.f. measured between a calomel half cell and a silver/silver chloride electrode showed a maximal rate of change/unit of silver nitrate added.

Mathematical treatment of the results

The percentages of Table 1 are expressed as mg. in the following calculation.

Correction for incomplete emptying of stomach

Concentration of phenol red in first recovery = 83·4 mg./l.
Concentration of phenol red in first washout = 10·0 mg./l.
Recovered volume of first washout = 0·190 l.
Amount of phenol red in recovered volume of first washout = 0·190 × 10 mg.
Volume of first recovery fluid equivalent to amount of phenol red in recovered washout

$$(V_{\mathrm{rem.}2}) = \frac{0{\cdot}190 \times 10}{83{\cdot}4} = 23 \text{ ml.}$$

The second washout contained a negligible amount of phenol red. In this way it was possible to estimate $V_{\mathrm{rem}\,2}$ with some precision.

Correction for gastric contents lost through the pylorus

Volume of ingested fluid containing phenol red and sulphaguanidine = 0·750 l.
Concentration of phenol red in ingested solution = 100 mg./l.
Amount of phenol red ingested = 0·750 × 100 mg.
$V_{\mathrm{rec.}} + V_{\mathrm{rem.}2}$ (see Table 1) = 0·515 l.
Concentration of phenol red in the sample first recovered = 83·4 mg./l.
Amount of phenol red recovered = 0·515 × 83·4 mg.

Amount of phenol red passing pylorus = amount ingested − amount recovered

$$= 0{\cdot}750 \times 100 - 0{\cdot}515 \times 83{\cdot}4 \text{ mg.}$$

Mean concentration of phenol red in gastric contents passing through the pylorus during the hour $= \frac{100 + 83{\cdot}4}{2} = 91{\cdot}7$ mg./l.

Volume of gastric contents passing through the pylorus

$$= \frac{\text{Amount of phenol red lost through pylorus}}{\text{Mean concentration of phenol red in gastric contents}}$$

$$= \frac{0{\cdot}750 \times 100 - 0{\cdot}515 \times 83{\cdot}4}{91{\cdot}7}$$

$$= 0{\cdot}349 \text{ l.}$$

Thus 0·349 l. of gastric contents passed through the pylorus.

Calculation of volume absorbed

By rearranging equation III,

$$V_{\text{abs.}} = V_{\text{in.}} + (V_{\text{sec.}} + V_{\text{rem.}_1}) - V_{\text{pyl.}} - (V_{\text{rem.}_2} + V_{\text{rec.}}). \tag{IV}$$

On the right side of the equation the only unknown is $(V_{\text{sec.}} + V_{\text{rem.}_1})$.

The calculation of $(V_{\text{sec.}} + V_{\text{rem.}_1})$ *from the calculated recovery of chloride and acid*

Concentration of chloride in first recovery = 29·14 m.equiv./l.

Amount of chloride recovered = 29·14 × 0·515 m.equiv.

Mean concentration of chloride in the 0·349 l. passing through the pylorus $= \frac{29{\cdot}14}{2}$ m.equiv./l.

Amount of chloride passing through pylorus $= \frac{29{\cdot}14 \times 0{\cdot}349}{2}$ m.equiv.

Total amount of chloride = amount recovered + amount lost through the pylorus = 20·09 m.equiv.

Similarly, the total amount of acid secreted may be calculated to be 11·25 m.equiv. From equation II it was found that this amount of Cl^- and acid corresponded to 131 ml. of gastric secretion, i.e. $V_{\text{sec.}} + V_{\text{rem.}_1} = 131$. Substituting numerical values in equation IV

$$V_{\text{abs.}} = 750 + 131 - 349 - 515,$$

therefore water absorbed = 17 ml., i.e. 2·7 % of the mean volume of the gastric contents.

Calculation of the amount of sulphaguanidine absorbed

Volume of test meal and secretion passing pylorus = 0·349 l. (see above).

Mean concentration of sulphaguanidine passing pylorus $= \frac{100 + 81{\cdot}1}{2}$ mg./l.

Amount of sulphaguanidine lost through pylorus $= \frac{0{\cdot}349 \times 181{\cdot}1}{2}$ mg.

Amount absorbed = amount ingested − amount recovered − amount passing pylorus

$$= 0{\cdot}750 \times 100 - 0{\cdot}515 \times 81{\cdot}1 - \frac{0{\cdot}349 \times 181{\cdot}1}{2} \text{ mg.}$$

$$= 1{\cdot}63 \text{ mg. of sulphaguanidine.}$$

Mean amount of sulphaguanidine in stomach

$$\frac{(0{\cdot}750 + 0{\cdot}515)}{2} \times \frac{(100 + 81{\cdot}1)}{2} = 57{\cdot}27 \text{ mg.}$$

Absorption of sulphaguanidine $= \frac{1{\cdot}63 \times 100}{57{\cdot}27} = 2{\cdot}8\,\%$.

Absorption of water (see above) = 2·7 %.

RESULTS

Nine experiments on eight healthy students progressed as far as the successful withdrawal and washout of the gastric contents; the results are given in Table 1. One other experiment during which the subject complained of nausea was discarded as the gastric contents were stained with bile. The mean calculated absorption of water was 9·3 ml./hr. S.E. of mean ± 4·03 ml. Expressed as a percentage of the mean volume of the gastric contents, a mean of 1·48 %, S.E. mean ± 0·63 % was absorbed. Calculated by the use of 'Student's' '*t*' test, the chance that this value would be found if there was in fact no water absorption is 1 in 20. The mean calculated absorption of sulphaguanidine expressed as a percentage of the mean amount of sulphaguanidine in the stomach was 1·41 %, S.E. mean ± 0·60 %. If there were no absorption of sulphaguanidine such a value might also be expected to arise once out of twenty times by chance. Thus considering the experiments as a group it appears that the absorption of water and sulphaguanidine are of the same order and are statistically significantly different from zero. However, when the values for each subject are considered separately, it is evident that no correlation between the absorption of sulphaguanidine and water has been demonstrated for individuals.

TABLE 1. The absorption of water and sulphaguanidine from the stomach of man

	No histamine				0·5 mg. histamine phosphate				
Subject ...	D.T.L.	D.T.L.	J.W.C.	W.V.J.	R.C.B.	E.P.T.	K.J.	I.M.	E.R.H.
Volume ingested (ml.)	750	750	750	750	750	800	600	600	500
Volume recovered (ml.) $V_{rec.} + V_{rem.2}$	530	705	599	515	776	766	649	588	523
Conc. phenol red recovered as % of conc. ingested	97·0	95·9	95·4	83·4	82·6	89·4	86·9	86·9	94·2
Conc. sulphaguanidine recovered as % of conc. ingested	94·0	92·6	93·7	81·1	83·9	88·3	87·5	86·9	91·6
Conc. Cl^- m.equiv./l. in recovered gastric content	9·80	7·30	5·12	29·14	29·62	13·56	23·88	23·37	8·29
Conc. acid m.equiv./l. in recovered gastric content	0·38	0·82	Nil	16·32	24·24	1·48	4·70	16·17	Nil
Volume gastric content passing through pylorus	240	75	183	349	119	122	39	95	8
Volume secreted calculated from amounts of Cl^- and acid secreted (ml.) $(V_{sec.} + V_{rem.1})$	47	39	26	131	151	82	114	93	33
Water absorbed (ml.)	27	9	−6	17	6	−6	26	10	2
Water absorbed expressed as % of mean vol. gastric contents	4·2	1·2	−0·9	2·7	0·8	−0·8	4·1	1·7	0·4
Sulphaguanidine absorbed expressed as % of mean amount in the stomach	3·1	3·4	1·8	2·8	−1·6	1·2	−0·7	0	2·7

DISCUSSION

Let us consider the sources of error in the calculations given for subject W.V.J.

(1) The calculation of the volume of secretion from the amounts of Cl^- and acid secreted has a mean error of ± 3·15 ml. and S.D. of an individual difference ± 2·54 ml.

(2) The sum of the standard errors of estimations of the concentrations of phenol red and sulphaguanidine have been shown to be ± 0·96 % (Hunt, 1947). If an error is made in the estimation of sulphaguanidine such that the true concentration is 82·1 mg./l. instead of 81·1 mg./l., the absorption of sulphaguanidine may be calculated to be 0·95 mg. This is 1·6 % of the mean amount of sulphaguanidine in the stomach instead of 2·8 % calculated from the results of the experiment. Thus an error of about 1 % in the estimation of the concentration of sulphaguanidine would in this case reduce the calculated absorption of sulphaguanidine by one-third.

(3) The assumption is implicit throughout that mixing in the stomach is complete. Although the sum of these errors is probably random about zero for a group of subjects, it was apparent that a considerable improvement in technique would be necessary to demonstrate any correlation between the absorption of water and sulphaguanidine in an individual. It was thought possible that the low rates of secretion seen in the first three experiments might be associated with a low gastric blood flow and that this might be a factor in reducing the absorption of water. For this reason a further five experiments were made with the addition of a subcutaneous injection of histamine. Whilst this was effective in causing an increase in the rate of secretion there was no apparent effect on the absorption of water.

It is perhaps worthy of note that subject R.C.B.D., who showed minimal absorption of water, had on two previous occasions been shown not to absorb sulphaguanidine when this was measured without the concurrent absorption of water being estimated. This accords with the experience of Wilhelmj, Finegan & Hill (1937) who found that individual dogs were consistent in their absorption or non-absorption of water. Thus, under the conditions of these experiments, water absorption of about 1·5 %/hr. appears to have taken place. This agrees well with the 6·5 %/3 hr. i.e. 2·2 %/hr., found by Sleeth & Van Liere (1937) in cats, especially if the relatively smaller surface area presented by the larger volume contained in the stomach of man is borne in mind.

The conditions of these experiments are unlike those existing in an actively motile digesting stomach, so that the results do not warrant the uncritical acceptance of the assumption that the stomach of man never absorbs significant quantities of water. Penner *et al.* (1940), who noted significant absorption of water, used much smaller volumes than were given in the present research. Corresponding to their findings, when smaller volumes of fluid were retained in the stomach during an earlier investigation of the absorption of sulphaguanidine (Hunt, 1947) it was found that up to 12% of the calculated mean total amount of sulphaguanidine in the stomach was absorbed. The olive oil instilled into the duodenum had a most remarkable delaying effect on gastric evacuation. Shay *et al.* (1939) found that olive oil instilled into the duodenum caused the stomach completely to retain 200 ml. of fluid for 30 min. It is, nevertheless, rather surprising that the same method should be so successful in inhibiting for 1 hr. the emptying of a stomach containing up to four times this volume.

SUMMARY

1. A technique is described for estimating the water and sulphaguanidine absorbed by the stomach.

2. In nine experiments on eight healthy human subjects the mean absorption of water was 1·5%/hr. of the mean volume of water in the stomach. The absorption of sulphaguanidine was 1·4%/hr. of the mean amount of sulphaguanidine in the stomach. These values are statistically significantly different from zero.

3. Olive oil instilled into the duodenum was in every case very effective in slowing the emptying of the stomach containing more than 500 ml. of test solution.

My thanks are due to the students who enthusiastically took part in these experiments. I am greatly indebted to Dr R. B. Fisher for advice and help with the statistical calculations and to Prof. W. R. Spurrell for encouragement and many helpful suggestions in the preparation of the manuscript.

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RESPONSE OF A FROG'S MUSCLE SPINDLE

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In 1931 Matthews described a nerve muscle preparation in the frog which contained a single end-organ. On stretching the muscle the frequency of nerve impulses set up by the end-organ was roughly a linear function of the logarithm of the load, i.e. the end-organ obeys Fechner's law.

The present experiments examine the application of Weber's law to the muscle spindle.

METHOD

The method differs slightly from the one described by Matthews. The musculus externus profundus II with the branch of the peroneal nerve supplying it, was isolated and placed in a small trough (Fig. 1). The proximal tendon of the muscle was fixed to the trough. The distal tendon was tied to a thread which was led over two pulleys to a weight standing on a small platform, held up by a trigger. When the trigger was released the platform fell away and the weight hung on the thread thus stretching the muscle. The muscle was submerged in oxygenated Ringer's solution run through the trough continuously. The cut end of the nerve was lifted out of the fluid and placed on an

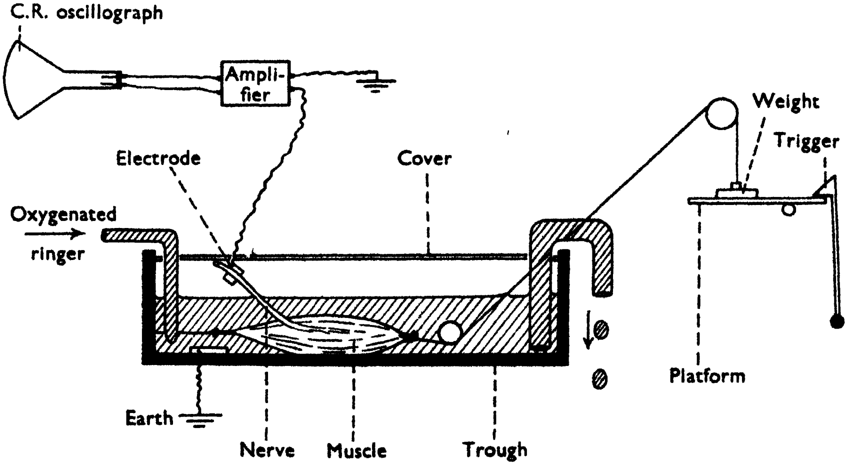

Fig. 1. Arrangement of preparation.

electrode, and the trough was covered to prevent drying. Another electrode connected the fluid to earth. The nerve action potentials were amplified and recorded by a cathode-ray oscillograph.

Procedure. The muscle was stretched by a certain weight and after an interval of 10 min. for adaptation the nerve response was recorded. Now an additional weight was applied and the response again recorded.

After 1–2 min. the additional weight was removed, and after waiting 5 min. was replaced by a heavier one. This procedure was repeated until a definite increase in frequency of impulses occurred. The muscle spindle adapted in about 3–4 min. but did not adapt completely.

A different behaviour, however, was observed sometimes when the muscle was loaded with a weight so big that an additional one might break the muscle. In these cases the frequency of impulses rose suddenly on applying the weight, but declined rapidly to nothing and no further response occurred. It is improbable that the muscle could be stretched so severely when *in situ*.

RESULTS

In general, the observations confirmed those of previous workers. When the muscle was unloaded the frequency of nerve impulses was 2–3 per sec. When loaded the impulse frequency rose steeply and fell slowly. By 4 min. a steady frequency was reached which was maintained for 50 min., but was 6–9 per sec. higher than in unloaded muscle.

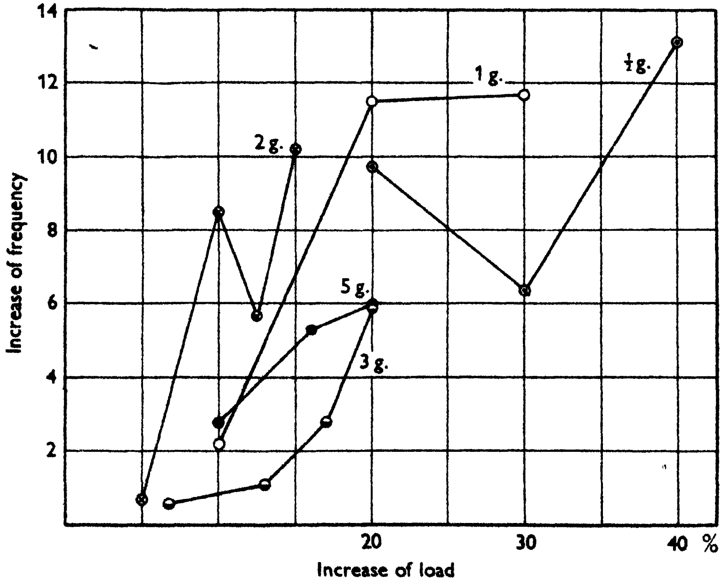

Fig. 2. Increase in impulse frequency (ordinates) when a steady load of 0·5, 1, 2, 3 or 5 g. suddenly received the percentage increase shown as abscissa.

The results of one experiment to test Weber's law are shown in Fig. 2. For instance, the black circles show the results with a steady load of 5 g. Additional weights of 0·5, 0·8 and 1·0 g. are plotted horizontally as percentage increase in load against the observed increase in response frequency per sec. (vertical). The frequency increase might be expected to be proportional to the load increase, and the 5 g. curve in fact lies close to a straight line through the origin. In the results with smaller loads random factors enter to a much greater extent, and so it seemed best to apply a statistical treatment. I am indebted to Dr W. A. H. Rushton and Mr A. F. Huxley for the method and formulae appropriate to the present case.

It is assumed (i) that there is no error in the load W or its increment ΔW, but that a random fluctuation affects the measurement of the frequency increment Δn; (ii) that the 'true' results should lie on a straight line through the origin

$$\Delta n = b\,\frac{\Delta W}{W}. \tag{1}$$

From this it follows that the 'best' value of b is given by

$$\frac{b}{W} = \frac{\Sigma(\Delta W, \Delta n)}{\Sigma(\Delta W)^2},$$

and the standard error of this estimate is

$$\sqrt{\frac{\dfrac{\Sigma(\Delta n)^2}{\Sigma(\Delta W)^2} - \dfrac{b^2}{W^2}}{r-1}},$$

where Σ is the sum of the values specified for each of the r points of the group.

Now if Weber's law is interpreted as stating that $\Delta W/W$ is constant for a fixed value of Δn, it follows from equation (1) that

$$b = \Delta n \Big/ \left(\frac{\Delta W}{W}\right) = \text{constant},$$

or b should be independent of the load. In Fig. 3 the value of b is plotted for each load W. The best value of b is given by the mid-point of each rectangle, whose vertical extent shows ± twice the standard error. The shaded rectangles correspond with the results of Fig. 2. It is seen that the value of the ordinate lies around 3 impulses/sec./10% increase in load, but that this figure will hardly satisfy all loads within experimental limits. It does not appear, however, that this is due to any consistent divergence from Weber's law, but rather that three or four determinations at each load (Fig. 2) are insufficient to give a reliable value of b. The white rectangles (Fig. 3) are from another experiment where loads were higher and about ten determinations contributed to each rectangle. In this case a value of 2·7 for b lies well within the experimental error of the three loads investigated.

These results suggest that Weber's law holds as a property of the single stretch receptor, but random fluctuations so invalidate single observations that the relation is only clear when a large number of results are taken into consideration. This no doubt is the case in conditions of sensory judgement where the law was originally formulated.

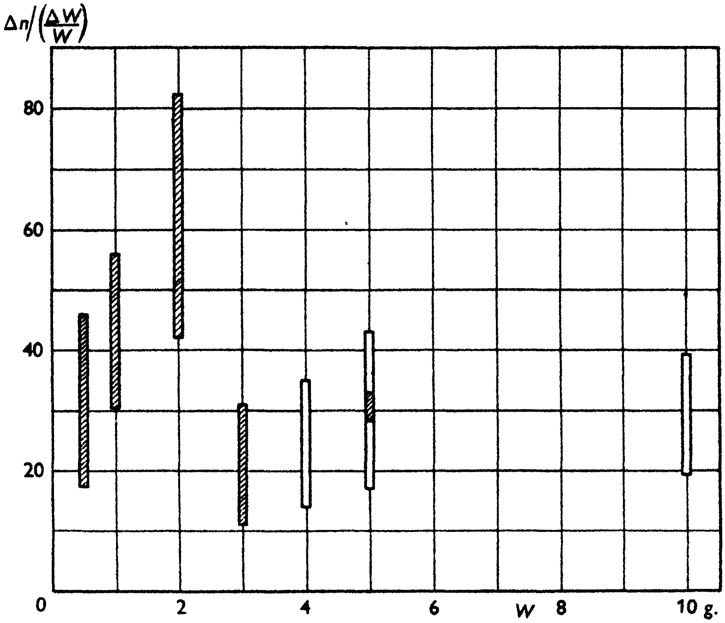

Fig. 3. Against each load (abscissa) is plotted the increase in frequency produced by a constant Weber fraction ($\Delta W/W$). Results obtained statistically, height of rectangles showing standard error. Shaded rectangles from Fig. 2, others from another experiment with far more observations contributing to each rectangle.

SUMMARY

1. The least additional weight required to produce a constant increase in response frequency in a single stretch receptor has been determined.
2. It is found that after adaptation a 10% increase in weight produces the same additional response, for a wide range of initial weights.
3. The relation of this to Weber's law is discussed.

It is a pleasure to thank Prof. Adrian and Dr Matthews for their help during this work, and to express my indebtedness to the Rockefeller Foundation for the financial assistance which made it possible.

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EXPLANTATION OF THE RIGHT KIDNEY IN THE DOG

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For the study of certain problems in renal physiology a preparation was required from which simultaneous collections of renal arterial blood, renal venous blood, and urine could be made, without interruption of the blood flow to the kidney whose function was under investigation. Could both kidneys be explanted, the one giving access to the renal artery, the other to the renal vein, it would be reasonable to assume that the composition of the arterial blood supplying the two kidneys is identical. Provided that the urine and the venous blood samples were collected from the same explanted kidney, the preparation should prove satisfactory.

Rhoads (1934*a*) published a method for the explantation of the left kidney in the dog which rendered the left renal vein readily accessible. He discussed the renal nerve supply, and showed how such preparations could be made with intact innervation. Two years after operation (Rhoads, 1934*b*) the dogs were healthy and the kidneys were functioning normally, but were altered in shape by the pressure of surrounding structures. Lockett, O'Connor & Verney (1942) described a method by which access was gained to the left kidney, the pedicle was exposed, and a renal artery loop made. If so desired the kidney was denervated at the time of the final operation.

The right kidney in the dog lies nearer the head than the left. Approached from the postero-lateral surface the right kidney is normally completely covered by the lower ribs, and the hilum is not infrequently opposite the tenth intercostal space. For the technique of explantation, the right kidney therefore presented new problems. These have been solved by subperiosteal resection of the lower ribs, coupled with the simple expedient of moving the posterior part of the diaphragm proximally. It has been found necessary to remove the lower four ribs in order that, when the animal turns briskly to look in the direction of its tail, the explanted kidney is not bruised by a projecting costal margin. The height to which the diaphragm must be moved varies with the individual animal, and is determined by direct observation at the time of operation.

TECHNIQUE

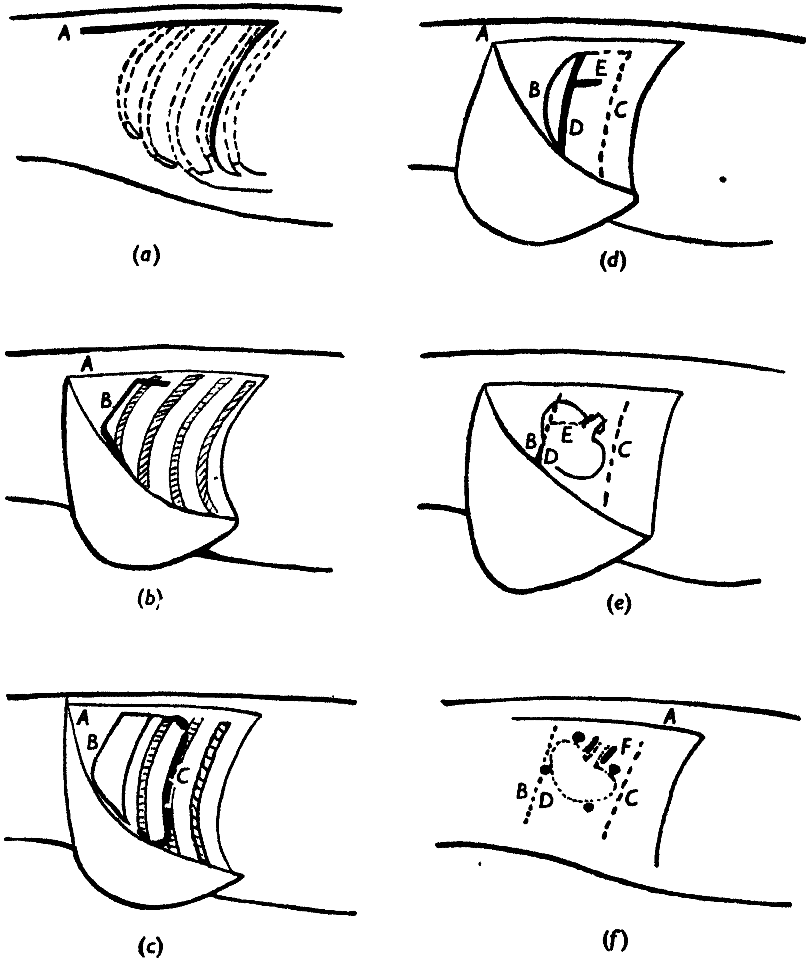
Healthy moderately large bitches, equipped with carotid loops (van Leersum, 1911) with the carotid sinus denervated for determinations of blood pressure (Verney & Vogt, 1938), perineotomized, trained, and with the left kidney explanted and having a renal artery loop with intact innervation (Lockett *et al.* 1942) were used. The dogs received 3 mg. of atropine sulphate subcutaneously, and were anaesthetized with intravenous thiopentane; a rubber, oral, cuffed MacGill tracheal tube* was inserted. Anaesthesia was maintained with cyclopropane oxygen mixture administered in a closed circuit. The animals were then placed on their left sides over a soft low sandbag, and were shaved from the 7th rib to the iliac crest. Asepsis was maintained.

The skin incisions extended along the upper border of the 9th rib from the costal cartilage backward to a point 1 cm. to the right of the spine, and thence vertically downward for a distance of 8–12 cm. The skin flap was reflected, and the lower four ribs removed subperiosteally. An incision was then made through the flank muscles 1 cm. below and parallel to the periosteum of the 12th rib. The position of the right kidney was viewed (Text-fig. 1*a* and *b*).

In the majority of animals the kidney was fairly readily accessible, and it sufficed to raise the diaphragm by interrupted suture to the level of the 10th rib periosteum (Text-fig. 1*c*), to fix it there more firmly by a continuous suture, then to free the inverted thoracic wall (Text-fig. 1*d*), drawing it down as a curtain to cover the kidney, and finally to bury the cut edge adjacent to the diaphragm by continuous suture. In one dog the kidney was very high, and for the full mobilization of the diaphragm the erector spinae muscles were freed from their attachment to the first lumbar transverse process, and the anterior and posterior divisions of the first lumbar artery were divided between ligatures. The upper part of the latissimus dorsi and the psoas were removed; the arcuate ligaments were mobilized by the subperiosteal resection of the first lumbar transverse process; the diaphragm was then stitched to the periosteum of the 9th rib.

The thoracic curtain was retracted, and the kidney gently freed from perirenal fat; the pedicle was cleaned without disturbing the nerve supply. The kidney was lifted outward, and was turned over posteriorly to expose the renal vein, and was allowed to rotate with respiration to a final position which it reached in 6 or 7 min. A vertical incision was made in the thoracic curtain which was sutured distal to the renal pedicle, beneath the kidney; the thoracic curtain was then sutured horizontally to the divided flank muscles forming a sling under the kidney (Text-fig. 1*e*).

The skin flap was drawn over the kidney, and was fixed firmly by suture from the inner surface over the renal poles (Text-fig. 1*f*), holding the kidney in position. A small flap was cut in the skin to cover the renal pedicle, and was sutured firmly on either side of the renal vessels and ureter to the thoracic sling, with strong silk. The vertical part of the skin incision was then sutured, followed by the thoracic part, and the outer edges of the pedicle flap. This



Text-fig. 1. Diagrammatic representation of the operation. (*a*) Skin incision *A*, followed by reflexion of skin flap. (*b*) Subperiosteal removal of lower four ribs, and incision below and parallel to 12th rib periosteum, *B*. (*c*) Periosteal thoracic wall turned inward and diaphragm sutured to 9th or 10th periosteum along its length, *C*. (*d*) Under surface of inverted thoracic wall cut free and drawn downward, leaving raised diaphragm. Vertical cut *E* in thoracic wall *D* to allow the passage of the renal vessels. (*e*) Kidney and pedicle freed; *E* sutured under the kidney; thoracic flap *D* sutured to the abdominal muscles *B*, forming a sling under the kidney. (*f*) Skin flap sutured over the renal poles: small pedicle flap *F* cut; incisions closed.

pedicle flap, corresponding to a similar one used by Rhoads, marked the position of the renal vein, and since sutures were left in for 2 weeks, caused scarring which helped to fix the pedicle in position. All other sutures were removed on the 6th day. Sterile dressings were applied, and changed at intervals of 2–3 days until all sutures had been removed (Pl. 1).

The photograph shows the explanted kidney in the final position reached after rotation in relation to the skin incisions.
The renal vein lies just beneath the pedicle flap.

Alternatively, the method described by Lockett *et al.* for the left renal artery loop in the dog can be extended and made suitable for right renal artery loops by the procedures described above.

Kidneys explanted by the method described have not altered in size or shape during the first few months of observation. The urines at no time contained albumen, blood, casts or pus. The non-protein nitrogen content of the blood and the creatinine clearance values remained unchanged.

SUMMARY

A method for the explantation of the right kidney of the dog into the flank is described: the high position of the right kidney in this animal necessitates the removal of the lower four ribs and the shortening of the thoracic cavity by raising the diaphragm. Such an operation makes satisfactory bilateral renal explant preparations possible in the dog.

A part of the expense of this work was defrayed by a grant from the Medical Research Council. I wish to thank my two technicians, Joan Nunn and Phyllis Harris, for their assistance at operations and dressings, and for their careful attention to the dogs.

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THE INHIBITION OF WATER DIURESIS IN MAN BY ISCHAEMIC MUSCLE PAIN

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It has frequently been observed that unpleasant sensory or emotional stimuli may inhibit water diuresis in dogs. Such stimuli include gentle restraint (Rioch, 1930), shaving the skin or lumbar puncture (Theobald, 1934), acupuncture (Theobald & Verney, 1935), raucous noise, unfamiliar exercise or weak faradic stimulation (Rydin & Verney, 1938). The phenomenon has been intensively studied by Verney and his collaborators (Klisiecki, Pickford, Rothschild & Verney, 1933; Theobald & Verney, 1935; Rydin & Verney, 1938; O'Connor & Verney, 1942, 1945; O'Connor, 1946; Verney, 1946, 1948). They have proved conclusively that the inhibition of diuresis so produced is of two distinct types. The first, in which the onset of inhibition is abrupt and the duration brief, is due to renal vasoconstriction: the second, in which the onset is slower and the duration more prolonged, is due to reflex liberation of posterior pituitary antidiuretic hormone.

In spite of the detailed investigation which has been made of this phenomenon in the dog, relatively little work appears to have been done to determine whether such mechanisms can be demonstrated in man. Exercise has indeed been shown to inhibit diuresis (MacKeith, Pembrey, Spurrell, Warner & Westlake, 1923; Hellebrandt, Walters & Miller, 1936; Eggleton, 1943) and to reduce renal blood flow (Barclay, Cooke, Kenney & Nutt, 1947): diminution in urine output, however, does not invariably accompany the reduction of renal blood flow (Barclay, Cooke, Kenney & Nutt, 1947), which suggests that it is not a purely vascular phenomenon. Inhibition of diuresis also follows syncope induced by passive support in the semi-erect position, and the evidence again suggests that this may be due to liberation of posterior pituitary antidiuretic hormone as well as to reduction in renal blood flow (Brun, Knudson & Raaschou, 1946). The effects of purely sensory stimuli have been studied by Wolf (1943), who induced severe pain by mechanical pressure on the scalp, and by Talso, Crosley & Clarke (1948), who induced pain by immersion of one foot in ice-water for 15 min. Both types of pain caused reduction of renal blood flow and of urine output: the possible rǫle of the neurohypophysis in the diminution of urine flow, however, was not specifically investigated.

In view of the many tests of renal function in common use for clinical or research purposes, in which diuresis is deliberately induced, it appeared to me that further evidence was desirable as to whether inhibition of diuresis by sensory stimuli could be convincingly demonstrated in man and if so, whether reflex stimulation of the neuro-hypophysis was involved. The sensory stimulus chosen was pain, induced by exercising muscles under ischaemic conditions, since this is simple, safe and subject to reasonably precise control. This paper records the results of preliminary experiments, which establish that inhibition of diuresis can be produced in man by this method and suggest that the inhibition is predominantly or wholly due to release of posterior pituitary antidiuretic hormone.

METHODS

The experiments were performed on ten healthy male subjects, all medical students or members of the staff of the Department of Medicine. Their ages ranged from 22 to 43 years. Experiments were done in the morning or afternoon, beginning at approximately 10 a.m. or 2 p.m., the subjects having finished breakfast or lunch at least 1 hr. previously. It was not considered practicable to attempt to establish a uniform level of hydration or salt balance before the experiments, since these had to be fitted into the subjects' working time as circumstances permitted.

At the start of each experiment, the subject emptied his bladder completely and drank 1 l. of tap water in 2–5 min. He then sat quietly, engaged in reading or conversation, and emptied the bladder at intervals of 5–20 min., the time of completion of micturition being taken to the nearest minute. Each specimen of urine was measured and a sample was kept for examination. Records of the pulse rate and blood pressure, measured in the left arm, were made at frequent intervals.

Pain was induced by strong gripping movements of the right hand, the circulation to the right arm being occluded by a sphygmomanometer cuff at a pressure of 180–200 mm. of mercury. The gripping movements were performed approximately once every 2 sec., and were continued until the aching pain thus produced became too severe for further muscular effort to be made: this stage was reached after 1½–2 min. Occlusion of the circulation to the right arm was then maintained for a further 7–13 min.: during this period the pain became increasingly difficult to tolerate, and was accompanied in all subjects by restlessness and slight sweating, and in some by nausea. In one subject, hyperpnoea occurred and produced mild tetany. Release of the cuff always relieved the pain within a few seconds. all subjects had very nearly reached the limit of voluntary endurance of pain when it was terminated.

The posterior pituitary extract used was 'Pituitrin' (Parke, Davis): dilute solutions, containing 0·01–0·05 unit/ml. and adjusted to a pH of 3–4 with a potassium acetate-acetic acid buffer solution, were prepared at regular intervals and stored in the refrigerator in ampoules containing 1–2 ml.

Urinary chlorides were estimated by Volhard's method.

RESULTS

Effect of pain on water diuresis. In all ten subjects, inhibition of diuresis followed a period of pain induced when the diuresis was well established. In one subject the degree of inhibition, though definite, was relatively slight: in the remaining nine, the degree of inhibition was profound, urinary output falling to a level similar to, or even below, the resting value. The diminution in urine flow occurred progressively over a period up to 20 min. after the relief of pain: the specimen passed immediately after release of the cuff, representing urine secreted during the period of pain, showed little or no fall in output, and the inhibition reached its maximum between 7 and 20 min. later. The output of urine then slowly rose to a second peak, usually lower than the first, 1–2 hr. later. The inhibition was accompanied by a striking increase in urinary pigment, by increase in specific gravity and by marked concentration of urinary chloride, which sometimes reached a level higher than in the resting specimen. Proteinuria was not observed. The result of a typical experiment is shown in Fig. 1.

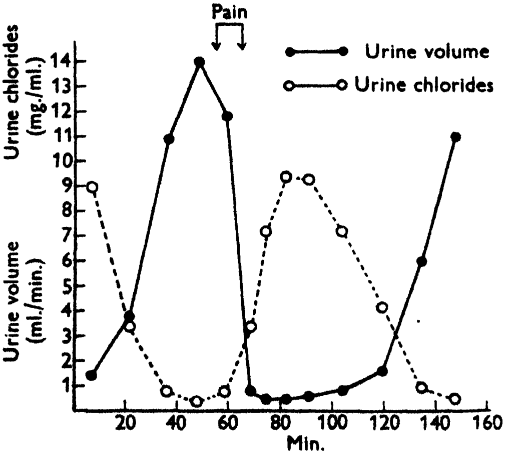

Fig. 1. Inhibition of diuresis by muscle pain. Subject L.K., aged 22 years. One litre of tap water drunk at zero time. Muscle pain induced during period shown by method described in text. In this and in all subsequent figures, urine volume and chlorides are charted at midpoint of corresponding period of collection.

Changes in pulse rate and blood pressure. In spite of the severity of the pain, changes in pulse rate were small and inconstant, the greatest increase in any subject being 16 beats/min. A rise in mean blood pressure, varying from 10 to 33 mm. of mercury, occurred during the pain in nine subjects, and a fall of 20 mm. of mercury in the tenth. Both pulse rate and blood pressure, however, rapidly returned to normal on relief of the pain, and had reached their resting levels several minutes before the urinary output had fallen to its lowest level (Fig. 2). It was clear that the inhibition of diuresis was not due directly to any general circulatory disturbance involving either heart rate or blood pressure.

Effects of simple circulatory arrest and of pain followed by occlusion of the venous return. It appeared possible that the inhibition of diuresis might be due to the formation of some antidiuretic substance in the tissues of the arm during circulatory arrest, rather than to the pain produced. However, no inhibition of diuresis occurred after simple occlusion of the circulation to the arm for periods of 10–40 min. in three subjects, nor after circulatory arrest combined with exercise of the muscles for a period insufficient to produce pain. As a further check, pain was terminated in four subjects by reducing the pressure in the cuff to 70 mm. of mercury, thus restoring arterial circulation to the limb while maintaining arrest of the venous return for a further period, which varied from 14 to 23 min. In each case, inhibition of diuresis occurred which in extent, rate of development and changes in urinary chloride was entirely comparable to that seen in experiments in which the venous return was not arrested, maximum inhibition being reached, in three of the experiments, while the venous tourniquet was still in place (Fig. 3). It was therefore concluded that the inhibition of diuresis was initiated by nervous impulses and not by the release into the blood of any substance formed in the tissues of the ischaemic arm.

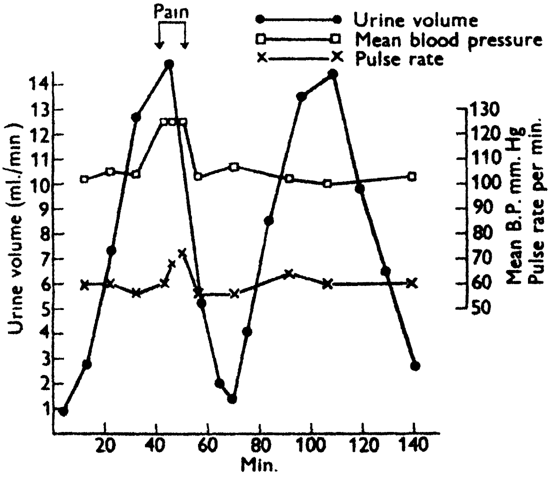

Fig. 2. Changes in blood pressure and pulse rate during inhibition of diuresis by muscle pain. Subject D.A.T., aged 22 years. One litre of tap water drunk at zero time. Muscle pain induced during period shown. Note that blood pressure and pulse rate have returned to resting level before inhibition of diuresis has reached its maximum.

Effect of adrenaline. Adrenaline is known to reduce renal blood flow in man, probably by causing vasoconstriction predominantly at the efferent glomerular arterioles (Chasis, Ranges, Goldring & Smith, 1938; Barclay, Cooke & Kenney, 1947), a site of action also suggested by experiments on the isolated kidney (Richards & Plant, 1922; Winton, 1931). However, doses up to 1·0 mg., subcutaneously or intramuscularly, or continuous intravenous infusions produce no constant effect on water diuresis in man (Theobald & Verney, 1935; Barclay, Cooke & Kenney, 1947), while large doses intravenously in dogs have either no effect on water diuresis (Theobald & Verney, 1935) or cause only transient inhibition of urine flow (Rydin & Verney, 1938). The slow development and prolonged duration of the inhibition in my own experiments and the accompanying concentration of urinary chloride and pigment made it improbable that the release of adrenaline during the period of pain played any significant part in the urinary changes. Nevertheless, it seemed necessary to examine this possibility, and in five subjects 1·2 mg. of adrenaline was injected subcutaneously or intramuscularly when diuresis was well established. This dose was sufficient in all cases to produce a rise in systolic blood pressure greater than that produced by muscle pain, but in one case a coincident fall in diastolic pressure caused the rise in mean pressure to be less than that produced by pain. Facial pallor, tremor, consciousness of the heart beat and sense of apprehension were all marked, and the subjects had no doubt that the subjective symptoms were greater than any experienced during periods of muscle pain: the rise in blood pressure, too, was of a different type, the systolic pressure rising far more than the diastolic, whereas during pain both systolic and diastolic pressures rose proportionately. Although there was a slight diminution in urinary flow after the injection of adrenaline in all five subjects, there was no inhibition of diuresis in any way comparable with that produced by pain and no significant change occurred in urinary chloride concentration. In one subject (A.R.K.) the effect of a considerably larger dose (1·75 mg. subcutaneously) was studied. This quantity was sufficient to raise the systolic blood pressure from 110 to 180 mm. of mercury and to cause very marked subjective and objective effects, including multiple extrasystoles: nevertheless, only brief inhibition of diuresis occurred, with very slight increase in urinary chloride concentration, the curves

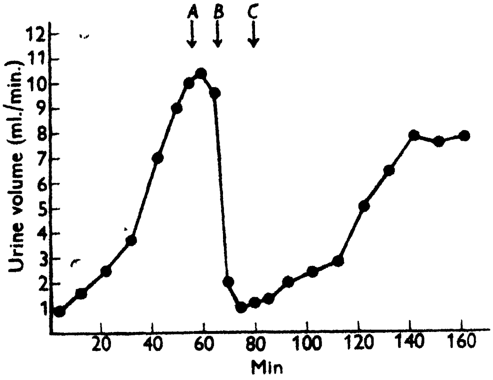

Fig. 3. Inhibition of diuresis by muscle pain followed by arrest of venous return. Subject A.R.K., aged 37 years. One litre of tap water drunk at zero time. Muscle pain induced during period *A* to *B*. At *B*, pressure in cuff reduced from 180 to 70 mm. of mercury. At *C*, cuff removed. Note that inhibition of diuresis is maximal before release of venous return from arm.

(Fig. 4) being quite unlike those obtained after muscle pain in the same subject (Figs. 3 and 5A, C). It may be said, therefore, that the inhibition of diuresis after pain cannot be wholly or predominantly due to adrenaline release, though the possibility cannot be excluded that this mechanism plays some part in the initial stages of inhibition.

Effect of intravenous injections of posterior pituitary extract. Posterior pituitary extract, in doses varying from 0·005 to 0·1 unit, was given intravenously during diuresis in thirteen experiments on seven subjects. Such doses did not affect the blood pressure and produced no subjective symptoms. The antidiuretic potency varied to some extent between different batches of extract, but the inhibition of diuresis so produced was always essentially similar, in onset, duration and urinary changes, to that produced by muscle pain, and in some experiments the curves of urinary volume and chloride concentration showed a close resemblance to those obtained after pain in the same or another subject (Fig. 5). As a more precise check, a comparison was made of the intervals between the time at which pain was ended or posterior pituitary extract was injected and the time of maximum inhibition of diuresis. The relief of muscle pain was taken as the point from which the time was measured on the assumption that, if the inhibition of diuresis were partly or wholly due to a reflex stimulation of the neuro-hypophysis, release of posterior pituitary hormone might be expected to occur predominantly when the pain was becoming almost intolerable and might continue for an unknown period after its relief. Only those experiments were included in which all periods of urine collection were less than 10 min. There were six such experiments on five subjects in whom diuresis was inhibited by pain, and nine experiments on six subjects to whom posterior pituitary extract was given. The results of the comparison were as

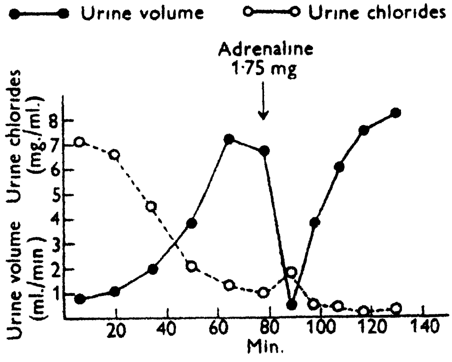

Fig. 4. Effect on diuresis of large dose of adrenaline. Subject A.R.K., aged 37 years. One litre of tap water drunk at zero time. 1·75 mg. adrenaline injected subcutaneously at point shown. Note brief inhibition of diuresis and small change in urinary chloride concentration. Compare with effect of muscle pain in same subject (Figs. 3 and 5, A, C).

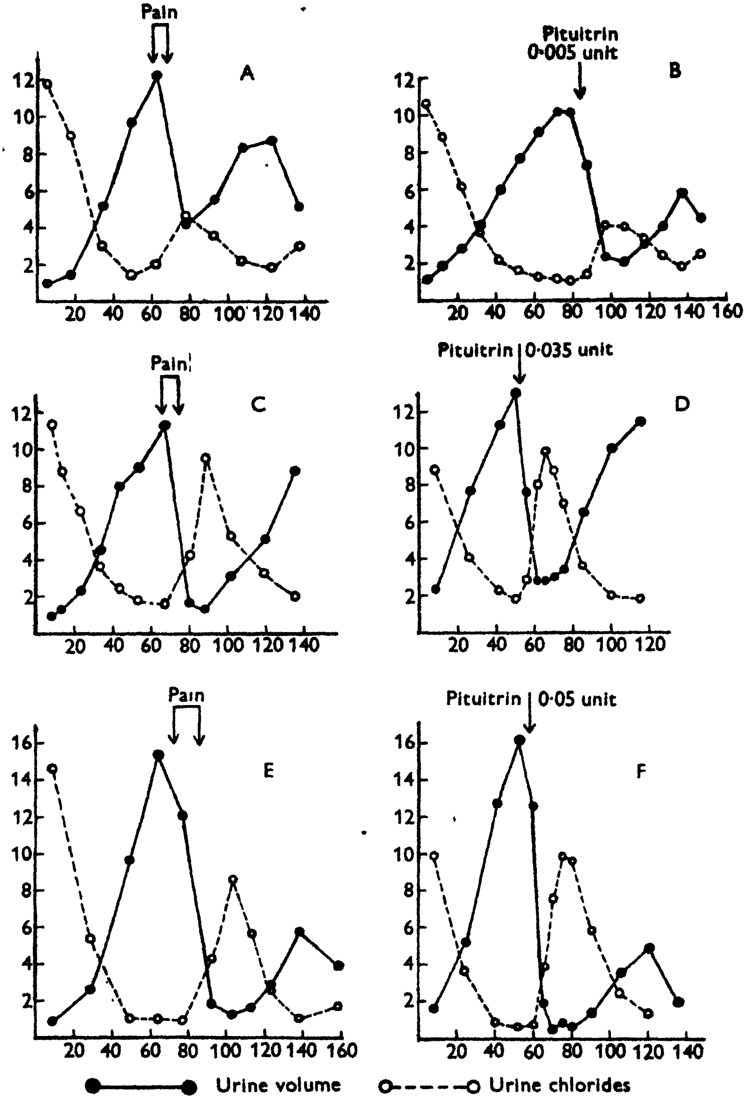

Fig. 5. Comparison of effects on diuresis of muscle pain and of intravenous posterior pituitary extract. Ordinates: urine volume in ml./min., urine chlorides in mg./ml. Abscissae: time in min. One litre of tap-water drunk at zero time in each experiment. A. Subject A.R.K. Effect of muscle pain for 7 min. B. Subject A.R.K. Effect of 0·005 unit posterior pituitary extract intravenously. C. Subject A.R.K. Effect of muscle pain for 9 min. D. Subject A.R.K. Effect of 0·035 unit posterior pituitary extract intravenously. E. Subject H.K.W. Effect of muscle pain for 13 min. F. Subject L.K. Effect of 0·05 unit posterior pituitary extract intravenously.

follows, the time of maximal inhibition being taken as the midpoint of the appropriate collection period:

Pain

Subject	Interval between relief of pain and maximal inhibition of diuresis (min.)
(1) A.R.K.	12·5
(2) A.R.K.	8·5
(3) D.A.T.	18·5
(4) J.W.S.	11·5
(5) L.K.	9·5
(6) R.E.H.P.	13·5

Range 8·5–18·5 min.
Mean 12·3 min.

Posterior pituitary extract

Subject	Dose (units)	Interval between injection and maximal inhibition of diuresis (min.)
(1) A.R.K.	0·01	11·5
(2) A.R.K.	0·02	14·5
(3) D.A.T.	0·03	13·0
(4) J.P.G.	0·035	12·0
(5) A.R.K.	0·035	11·0
(6) L.K.	0·05	13·5
(7) A.R.K.	0·07	17·5
(8) R.E.H.P.	0·1	14·0
(9) H.K.W.	0·1	23·0

Range 11–23 min.
Mean 14·4 min.

It is clear without detailed analysis that the interval between relief of pain and maximal inhibition of diuresis is of the same order as that between the intravenous injection of posterior pituitary extract and maximal depression of urine flow, and this agreement affords further support for the hypothesis that the inhibition of diuresis after pain is predominantly due to release of posterior pituitary hormone.

Effect of pain after injection of adrenaline. It was found by O'Connor & Verney (1945) that, in dogs previously subjected to renal denervation and section of the splanchnic nerves and abdominal sympathetic chains, the injection of adrenaline immediately before the application of an emotional stimulus usually abolished the slow type of inhibition of diuresis which previous work had established as being due to release of posterior pituitary hormone (Theobald & Verney, 1935; Rydin & Verney, 1938; O'Connor & Verney, 1942). Adrenaline, however, did not abolish the response to injected posterior pituitary extract, and it therefore seemed that release of antidiuretic hormone from the pituitary could be inhibited by adrenaline injection; the action is not specific, since tyramine in equipressor doses is just as effective, and it seems probable that it can be explained in terms of increase in sympathetic activity and cerebral blood flow (Verney, 1946). Since the inhibition of diuresis occurring in man after a period of pain was found to resemble closely that following the intravenous injection of posterior pituitary extract, it seemed relevant to investigate the effect on diuresis of a period of pain immediately preceded by an injection of adrenaline. This procedure was accordingly performed on five subjects, all of whom had shown marked inhibition of diuresis after pain alone: the dose of adrenaline was 1·2 mg. in each case, given subcutaneously in three experiments and intramuscularly in two, and the period of pain began 4–10 min. later. In R.E.H.P., J.W.S. and A.R.K. inhibition of diuresis occurred which was comparable in extent and duration to that produced by pain alone; in L.K. inhibition occurred, but was less in extent and slower in development than after pain alone; in D.A.T. no inhibition at all occurred, though the pain was fully as severe as in the experiment without adrenaline; it may be significant that D.A.T. was unusually sensitive to adrenaline, developing very severe tremor and tachypnoea though the rise in blood-pressure was less than in the other subjects. It thus appears that in certain persons a previous injection of adrenaline may modify or abolish the inhibition of diuresis produced by muscle pain, and this provides a further point of evidence in favour of the hypothesis that such inhibition is due mainly to release of posterior pituitary hormone and not to renal vascular changes.

DISCUSSION

These experiments have shown that water diuresis can be inhibited in man by a period of pain produced by exercising the forearm muscles after arrest of the circulation to them; that changes in pulse rate and blood pressure are of brief duration and have subsided before the inhibition of diuresis is fully established; that the inhibition is not due to the release into the blood of any antidiuretic substance from the arm; that the inhibition is accompanied by marked increase in urinary pigment and concentration of urinary chloride; that such inhibition cannot be produced by the subcutaneous or intramuscular injection of large doses of adrenaline, but can be closely paralleled by the intravenous injection of small doses of posterior pituitary extract; and that the inhibition may be modified or abolished in some subjects by a previous injection of adrenaline.

These results are all compatible with the hypothesis that the inhibition of diuresis produced in man by muscle pain is wholly or predominantly due to release of posterior pituitary antidiuretic hormone, and they are difficult to reconcile with any alternative theory. Apprehension or pain have been shown to reduce renal blood flow in man (Smith, 1939–40; Wolf, 1943), apparently by causing vasoconstriction chiefly at the efferent glomerular arterioles, since the filtration fraction rises under these circumstances; this action is, however, similar to that of adrenaline (Chasis *et al.* 1938; Barclay, Cooke & Kenney, 1947) which, as has been shown, does not cause any constant inhibition of diuresis or concentration of urinary chloride. It is indeed difficult to picture any purely vascular mechanism which would produce such striking evidence of change in renal tubular function. A vascular short-circuit through the juxtamedullary glomeruli, such as has been demonstrated in rabbits by Trueta, Barclay, Franklin, Daniel & Prichard (1947), has not yet been proved to occur in any other species, though the occasional autopsy finding of bilateral cortical necrosis of the kidneys suggests strongly that this mechanism may operate in man under certain circumstances; however, the types of stimuli required to produce it in the rabbit were all of an artificial or severely traumatic nature, and there is as yet no evidence as to the precise urinary changes which accompany this reaction. It cannot be denied that part of the inhibition during the period immediately following muscle pain may be due to vascular reactions within the kidney, and in view of the work of Wolf (1943) it is likely that such mechanisms are involved, but it seems improbable that the entire phenomenon can be so explained.

Assuming that the inhibition of diuresis after muscle pain is not wholly due to renal vascular changes, release of posterior pituitary hormone appears to be the most likely explanation: no other antidiuretic substance having the same type of action has ever been demonstrated in man, and the curves of inhibition of diuresis by pain in many of my experiments, allowing for the less frequent periods of urine collection, are strikingly similar to those given by Rydin & Verney (1938) and O'Connor & Verney (1942) as illustrating the slow type of inhibition produced in dogs by sensory stimuli and proved to be due to release of posterior pituitary hormone. It is true, as Verney (1946) pointed out, that 'a resembling relationship between phenomena does not carry the conviction of causal identity'; experiments on man, however, are of necessity limited in their scope, and accumulation of circumstantial evidence must often replace the direct proof which may be obtained by animal experiments.

Note added in proof. After this paper had been submitted for publication, my attention was drawn to the fact that adenosine triphosphate (A.T.P.), a substance known to be formed in ischaemic muscle, had been shown to possess an antidiuretic action (Green & Stoner, 1944); and that my experiments had not completely excluded the possibility that this or some similar substance might be responsible for my results, since blood might leave the limb even against a pressure of 70 mm. Hg if the local venous pressure rose to that level. Further experiments were therefore done on myself with this suggestion in mind. It was found possible, by varying the number and rate of muscular contractions, to induce a degree of pain which, though severe, was tolerable for periods up to 18 min., during which time several urine specimens could be collected: under these conditions, urine specimens passed towards the end of the period of pain showed definite diminution in volume and increase in chloride concentration, although the arterial circulation to the limb was still occluded. Moreover, in experiments in which Dr J. F. Goodwin and Dr A. G. MacGregor kindly co-operated, intravenous injections of the magnesium salt of A.T.P., given at the height of diuresis in doses up to 4·7 mg. in 17 sec., caused no significant inhibition of diuresis or change in urinary chloride concentration. This dose was sufficient to produce recognizable subjective symptoms, a fall of 20 mm. Hg in systolic blood pressure and temporary prolongation of the P-R interval in the electrocardiogram. I conclude therefore that the inhibition of diuresis by ischaemic muscle pain is initiated by nervous impulses and that it is not due to the release of A.T.P. from the ischaemic muscles.

SUMMARY

1. Inhibition of water diuresis was produced in ten male subjects by periods of pain, varying from 7 to 13 min., induced by exercising ischaemic muscles. The inhibition is of gradual onset and prolonged duration and is accompanied by concentration of urinary chlorides and increase in urinary pigment.

2. Changes in pulse rate and blood pressure are of brief duration and have subsided before the inhibition of diuresis is fully established.

3. The inhibition is not due to release of any antidiuretic substance from the ischaemic muscles.

4. Large doses of adrenaline do not produce comparable inhibition of diuresis.

5. Intravenous injections of small doses of posterior pituitary extract produce inhibition of diuresis which closely resembles that following pain as regards time relationships, increase in urinary pigment and concentration of urinary chloride.

6. In some subjects a previous injection of adrenaline may modify or abolish the inhibition of diuresis following muscle pain.

7. These results are all compatible with the hypothesis that in man, as in the dog, unpleasant sensory stimuli are capable of inhibiting water diuresis by reflex stimulation of the neuro-hypophysis.

I wish to express my thanks to all those who acted as subjects for these experiments; to Miss K. M. Radcliffe, who prepared the dilute solutions of 'pituitrin'; and to Drs A. E. W. Brooker, A. Dick and A. Jordan, who kindly assisted with injections or blood-pressure readings in the experiments on myself.

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THE ANTIDIURETIC AND OXYTOCIC HORMONES IN THE POSTERIOR PITUITARY GLANDS OF NEWBORN INFANTS AND ADULTS

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Newborn infants pass normally a very dilute urine (McCance & Young, 1941; Heller, 1944) and there is the suggestion that they are unable to concentrate it to the same degree as adults, even when fluid intake is restricted as during the first 3 days after birth. It has been pointed out (Heller, 1944) that glomerular filtrate of the composition elaborated by the kidney of the newborn and flowing at the low velocity resulting from the low glomerular filtration rate, would invariably be highly concentrated when passing through the tubules of a normal adult kidney. It would seem, therefore, that the neurohypophysio-renal mechanism which in the adult is the major determinant of the final concentration of the urine, operates differently in the newborn. The finding (Heller, 1944) that intramuscular doses of posterior pituitary extract which produced a pronounced inhibition of water diuresis in adults, had only a slight effect on newborn infants, agrees with this assumption. However, it still remains to be seen whether lack of antidiuretic hormone is a factor additional to the lower sensitivity of the tubules in preventing the newborn child from concentrating the urine like an adult.

It has been shown in a previous paper (Heller, 1947) that the pituitary gland of newborn rats (in which withdrawal of fluid even for 24 hr. fails to raise the concentration of the urine significantly (Heller, 1949)) contains only about one-tenth of the antidiuretic principle found in the glands of adults of the same strain. However, human beings are more mature at birth than rats and one would therefore expect to find more hormone in the posterior pituitary gland of newborn infants. The work described below gives the results of estimations of the antidiuretic and oxytocic hormones in the posterior pituitary lobes of fifteen newborn infants and fourteen adults.

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* British Council Scholar.

METHODS

Age, sex and cause of death of the subjects from which pituitary glands were obtained will be seen from Tables 1 and 2. The bodies were kept in refrigerators up to the time of the post-mortem examination. As soon as possible after the skull had been opened the hypothalamic region including the bones forming the pituitary fossa were removed 'en bloc' and were placed in a large weighing bottle kept in ice in a Dewar flask.

Preparation of extracts. With the help of low magnification the posterior lobe was dissected free from other tissue and immediately weighed in a tared weighing bottle. Five c.c. of acetone were then added which were renewed after 6 hr. After further 18 hr. the acetone was discarded and the gland dried first over calcium chloride and then above phosphorus pentoxide. Extracts were made by macerating the glands in a solution containing 0·9 % NaCl and 0·25 % acetic acid. For each mg. of dry gland tissue 0·5 c.c. was allowed. The extracts were boiled for 3 min., were then filtered and the residue washed five times with about 0·5 c.c. of 0·9 % NaCl or Burn-Dale solution (British Pharmacopoeia, 1932).

Assay of antidiuretic activity. Intravenous injections into unanaesthetized rabbits were employed (Heller, 1941).

Assay of oxytocic activity. Virgin guinea-pig uteri were used. Posterior pituitary extract (British Drug Houses) was used as the standard preparation.

RESULTS

The hormone content of the posterior pituitary lobe of newborn infants and adults. Tables 1 and 2 show the results of hormone estimations in the extract of individual glands. Table 3 gives mean results and their standard errors. No evidence could be obtained from our limited series for a sex difference in either the weight or the hormone content of the adult posterior pituitary lobes. The mean weight of adult posterior lobes (wet) of adult men was 139·1 ± 9·9 mg. (S.E. of eight observations) and that of adult women 131 ± 7·4 (5) mg. ($t = 0·63$, $P > 0·5$). The mean content of oxytocic hormone per mg. neurohypophysial tissue was 746 ± 64 (8) mU. in men and 748 ± 69 (5) mU. in women ($t = 0·03$, $P > 0·9$). The distribution of the sexes did not permit a similar calculation for the glands of newborns.

Influence on hormone content of the delay in removing the pituitary gland after death. The period of delay in removing the glands varied from 7·5 to 40 hr. after death (Tables 1 and 2). However, no significant correlation could be found between the length of time for which the gland remained in the dead body and the amounts of hormone found. For example, 'r' for oxytocic activity per mg. dry posterior lobe tissue of adults = $+0·47 \pm 0·29$, 'r' for oxytocic activity per mg. of dry posterior lobe tissue of newborns = $-0·33 \pm 0·29$. These figures have not been taken to mean that no losses of hormone occur after death, but only as an indication that such losses, if they occur, are within the limits of individual variations and of the errors of the methods of assay.

Dissection of the posterior pituitary lobe of newborn infants. The small size of the posterior lobe of newborn infants (mean weight = 15·1 ± 1·6 mg.) made its dissection difficult, and the question arose whether we succeeded in separating

Table 1. The antidiuretic and oxytocic hormone content of posterior pituitary glands of adults

No.	Age in years	Sex	Wt. of anterior lobe (wet, mg.)	Wt. of posterior lobe (wet, mg)	Wt. of posterior lobe as % of whole pituitary gland	Wt. of posterior pituitary lobe (dry, mg.)	Solids in posterior lobe (%)	Antidiuretic activity		Oxytocic activity		Delay in removal of pituitary gland (hr. after death)	Cause of death
								mU./posterior lobe	mU./mg. dry posterior lobe tissue	mU./posterior lobe	mU./mg. dry posterior lobe tissue		
1	14	♀	—	122·0	—	17·6	14·4	$\simeq 17600$	$\simeq 1000$	$\simeq 16000$	$\simeq 910$	13	Severe anaemia
2	15	♂	427·4	94·4	18·1	15·2	16·1	$>12500<16700$	$>830<1100$	$\simeq 15900$	$\simeq 1050$	22	Tuberculous meningitis
3	24	♂	—	140·7	—	17·0	12·1	$>6800<13600$	$>400<800$	$\simeq 9000$	$\simeq 530$	14	Chronic glomerulonephritis
4	32	♂	352·5	171·6	32·7	33·8	19·7	$\simeq 2000$*	$\simeq 60$*	$\simeq 680$*	$\simeq 20$*	7	Meningioma
5	39	♀	505·3†	123·3	19·6	19·1	15·5	$>9600<14300$	$>500<750$	$\simeq 9200$	$\simeq 510$	7	Chronic nephritis and hypertension
6	46	♂	276·8	126·2	31·3	17·5	13·9	$>8800<12400$	$>500<710$	$\simeq 11700$	$\simeq 670$	30	Lobar pneumonia
7	47	♂	408·4	166·1	28·9	22·0	13·3	$>11000<22000$	$>500<1000$	$>13900<27500$	$>630<1250$	24	Brain tumour
8	48	♂	482·6	149·9	23·7	25·2	16·7	$>18300<24000$	$>730<980$	$\simeq 16400$	$\simeq 650$	18	Shock
9	50	♂	—	111·0	—	17·3	15·7	$\simeq 6900$	$\simeq 400$	$\simeq 9900$	$\simeq 570$	15	Rectal carcinoma
10	51	♂	540·6	101·0	15·7	12·2	12·1	$>7600<9200$	$>500<750$	$\simeq 9800$	$\simeq 800$	25	Gastric ulcer
11	59	♀	548·1	143·3	20·7	20·0	14·0	$>19000<29000$	$>950<1450$	$\simeq 17000$	$\simeq 850$	24	Pulmonary embolism
12	60	♀	505·8	153·2	23·2	23·5	15·4	$>12900<17600$	$>550<750$	$\simeq 16400$	$\simeq 700$	20	Cerebral embolism
13	63	♂	197·4	124·2	38·6	18·6	15·0	$>14300<18600$	$>770<1000$	$\simeq 14100$	$\simeq 760$	26	Pneumonitis
14	64	♀	536·0	113·1	17·4	18·0	15·9	$>13500<18000$	$>750<1000$	$\simeq 13900$	$\simeq 770$	22	Pulmonary embolism

* Not included in mean (Table 3).

† Content of colloid cyst weighing 88·7 mg. not included.

Table 2. The antidiuretic and oxytocic hormone content of posterior pituitary glands of newborn infants

No.	Extra-uterine life (days)	Maturity (weeks)	Sex	Body wt. (kg.)	Wt. of anterior lobe (wet, mg.)	Wt. of posterior lobe (wet, mg.)	Wt. of posterior lobe as percentage of whole pituitary gland	Wt. of posterior lobe (dry, mg.)	Solids in posterior lobe (%)	Antidiuretic activity		Oxytocic activity		Delay in removal of pituitary gland (hr. after death)	Cause of death
										mU./posterior lobe	mU./mg. dry posterior lobe tissue	mU./posterior lobe	mU./mg. dry posterior lobe tissue		
1	Stillborn	40/40	♂	4·63	95·6	24·7	20·1	3·0	12·1	<1500*	<500	>990 <1850	>330 <650	7·5	Intracranial haemorrhage
2	Stillborn	Premature	♂	—	31·6	18·0	36·3	2·5	13·8	≃200	≃80	≃200	≃80	12·0	Prematurity
3	Stillborn	—	♀	—	48·4	22·2	31·4	2·2	10·0	≃400	≃180	≃320	≃150	24·0	Erythroblastosis foetalis
4	0·1	30/40	♀	—	54·2	7·9	12·8	1·8	22·8	>200 <330	>100 <180	≃210	≃110	9·5	Intracranial haemorrhage
5	0·1	—	♂	—	63·5	11·9	15·8	2·3	19·3	>400 <650	>175 <280	—	—	26·0	Cystic kidneys
6	1·0	28/40	♂	1·37	51·6	18·7	26·6	2·4	12·9	>240 <480	>100 <200	≃190	≃80	40·0	Prematurity, atelectasis
7	2·0	33/40	♂	2·31	41·6	14·4	25·7	3·0	20·8	>300 <600	>100 <200	≃240	≃80	12·0	Atelectasis
8	4·0	36/40	♂	1·98	45·5	15·2	25·0	1·7	11·2	>160*	>95*	≃160	≃95	7·5	Shock (P.M. : N.A.D.)
9	4·0	40/40	♂	2·66	74·2	20·4	21·6	2·0	14·2	>250 <500	>125 <250	>250 <480	>125 <240	20·0	Haemorrhagic disease
10	4·0	32/40	♂	1·47	53·4	13·7	20·4	2·8	20·4	≃200	≃70	—	—	14·0	Basal atelectasis. Kern icterus
11	16·0	38/40	♂	—	78·1*	29·5*	27·4*	4·2	14·2	≃500	≃115	>440 <580	>110 <140	30·0	Intracranial haemorrhage
12	26·0	40/40	♀	—	85·6*	9·0*	10·5*	1·5*	16·5	>400 <650	>270 <430	≃250	≃165	24·0	Congenital heart disease

* Not included in mean (Table 3).

it quantitatively from the rest of the gland. The antidiuretic and oxytocic activities of extracts from three whole pituitary glands were therefore estimated. Results of animal experiments (Heller, 1941, 1947) had shown that the presence of pars glandularis tissue did not interfere with the assay for the antidiuretic principle. The pituitary gland of the 1st infant of this series (age in days $=0{\cdot}1$, maturity $=28/40$) contained $\simeq 250$ mU. in terms of antidiuretic activity and $\simeq 300$ mU. in terms of oxytocic activity, that of the second infant (age in days $=0{\cdot}7$, maturity $=36/40$) contained $\simeq 330$ mU. and >250 <275 mU., and that of the third infant (age in days $=7{\cdot}0$, maturity $=28/40$) $>200<300$ mU. and >250 <375 mU. These figures compare well with the results obtained from separated posterior lobes (Table 2). They suggest that the dissection of the posterior lobe of newborn infants was successful.

TABLE 3. The antidiuretic and oxytocic hormone content of human posterior pituitary glands (means and their standard errors)

	Adults	Newborn infants
Wt. of anterior lobe (wet, mg.)	435 ± 33·8	60 ± 5·9
Wt. of posterior lobe (wet, mg.)	131 ± 6·5	15 ± 1·6
Wt. of posterior lobe as percentage of whole pituitary gland	24·5 ± 1·82	23·6 ± 2·22
Wt. of posterior lobe (dry, mg.)	19·8 ± 1·4	2·4 ± 0·2
Solids in posterior lobe (%)	14·9 ± 0·53	15·8 ± 1·38
Antidiuretic activity:		
(*a*) (mU./posterior lobe)	14570 ± 1580	375 ± 40
(*b*) (mU./mg. dry posterior lobe tissue)	761 ± 58	166 ± 25
Oxytocic activity:		
(*a*) (mU./posterior lobe)	13850 ± 1085	387 ± 20
(*b*) (mU./mg. dry posterior lobe tissue)	747 ± 45	150 ± 38

Proportion of antidiuretic to oxytocic activity in the posterior pituitary lobe extracts. No significant difference between the mean amounts of antidiuretic and oxytocic hormone was found in either the glands of adults (t between mU. antidiuretic and mU. oxytocic principle per mg. dry neurohypophysial tissue $=0{\cdot}19$, $P>0{\cdot}8$) or those of newborn infants ($t=0{\cdot}37$, $P>0{\cdot}7$). It seems, therefore, that in human posterior pituitary glands the two activities occur in the same proportion as in the international standard powder which is prepared from pituitaries of cattle or pigs.

Comparison of the hormone content of the posterior pituitary lobe of newborn infants with that of glands of adolescents and adults. The number of 'adult' glands (Table 1) investigated was too small to permit a subdivision into age groups. The mean hormone content of the pituitary lobes of two adolescents, aged 14 and 15, was slightly higher (antidiuretic activity per mg. dry tissue $=985$ mU., oxytocic activity $=980$ mU.) than the mean of adult subjects but this may have been accidental ($t=2{\cdot}12$, $P>0{\cdot}05$). When figures for all subjects between the ages of 14 and 64 (Table 1) were pooled, the following means and standard errors were obtained: antidiuretic activity per mg. dry neurohypophysial tissue = 761 ± 58 mU., oxytocic activity per mg. tissue = 747 ± 45 mU. The corresponding figures per mg. of dry neurohypophysial tissue of newborn infants were 166 ± 25 and 150 ± 38 mU. Per mg. dry tissue the posterior pituitary lobe of the newborns contained, therefore, approximately one-fifth of the active principles found in the glands of adults. Since the percentage of solids of the posterior lobe was found to be much the same in adults (14·9 ± 0·53 g./100 g.) and in newborn infants (15·8 ± 1·38, $t = 0{\cdot}69$, $P > 0{\cdot}5$), the ratio remains the same if hormone content is calculated per mg. *wet* neurohypophysial tissue.

When related to body weight the posterior lobe of newborns was found to weigh about four times more than that of adults (8·3 ± 1·2 mg./kg. body weight as against 2·0 mg./kg., assuming a mean body weight of 65 kg. for adults), while the ratio weight of posterior lobe/weight of anterior lobe was very similar in both instances (Table 3). Since, per mg. neurohypophysial tissue, the posterior lobe of newborn infants was shown to contain about one-fifth of the hormones found in that of adults, it follows that the hormone content of the posterior pituitaries of newborns, when calculated per kg. body weight, is only a little lower than that of adults. The following mean values were obtained for antidiuretic activity (oxytocic activity in parentheses): newborn infants = 185 ± 21 (175 ± 31) mU./kg. body weight, adults = 224 (213) mU./kg. body weight. It may be doubted whether much significance should be attributed to this relationship, which basically only expresses the high relative weight of the brain and its appendages in the human newborn.

DISCUSSION

While the active principles in the posterior pituitary gland of newborn infants seem to have been estimated only in isolated cases, several authors have reported on the hormone content of large series of adult human glands: Unfortunately, Lampe's (1926) careful work was done before the adoption of the international standard, which makes his results difficult to interpret. More recent is the work of Simon & Nagy (1934) who assayed the pressor and oxytocic principles, and that of Jores & Zschimmer (1934) who estimated the oxytocic principle only. The results of Simon & Nagy are in good agreement with ours. Their series of twenty adult pituitaries, for instance, contained a mean of 10750 ± 1721 mU. oxytocic hormone/gland; the mean of our estimations (derived from a comparable series) is 13850 ± 1085 mU./gland. The figures of Jores & Zschimmer are unaccountably lower.

The posterior lobe of newborn infants contained only about one-fifth of the antidiuretic and oxytocic principles found in the adult glands (per mg. dry tissue). This low hormone content is unlikely to have been due to an increase of secretory activity immediately before or during parturition: the hormone concentration (=mU./mg. neurohypophysial tissue) was much the same in newborn infants as in children who died 7 and 16 days after birth. It seems reasonable to assume that this interval would allow for replenishment of the gland. However, we may regard the neurohypophysis as modified hypothalamic tissue (see, e.g., Griffith, 1940), i.e. as bearing a similar relationship to the hypothalamus as does the adrenal medulla to the sympathetic nervous system. The low hormone content of the posterior pituitary lobe of newborn infants would thus be well in harmony with the immaturity of the human hypothalamus at birth (Le Gros Clark, 1938; Papez, 1940).

The demonstration, in the newborn child, of a low concentration of antidiuretic hormone in the posterior pituitary gland, leads to the question whether the apparent inability of newborn infants to concentrate the urine to the same degree as adults arises from a lower sensitivity of their renal tubules to the antidiuretic hormone (Heller, 1944) or whether lack of hormone production contributes to this inadequacy of the neurohypophysio-renal mechanism at birth. It was shown (Table 3) that the content of antidiuretic hormone of the neurohypophysial tissue of newborns was about 20% of that of adults. The hormone content of the posterior lobe of a boy of 10 suffering from diabetes insipidus, which we recently had occasion to investigate (details of this case will be given in a later publication) was less than 1% of the normal, a finding which is in good agreement with estimations of the antidiuretic hormone in the gland of experimental animals whose pituitary stalks had been severed (Fisher & Ingram, 1936; Hickey, Hare & Hare, 1941; Hare, Hickey & Hare, 1941). Conversely, it has been shown by O'Connor & Verney (1942) in dogs that a residuum of about 5% of the antidiuretic function of the neurohypophysis suffices to constrain the output of urine within normal limits. While the hormone content of the neurohypophysial tissue of the newborn infant is undoubtedly much lower than in the adult, it is thus hardly low enough to attribute the low concentrations of urine in newborn infants to a deficiency of the antidiuretic pituitary principle.

SUMMARY

1. Antidiuretic and oxytocic hormones were estimated in the posterior pituitary lobes of fourteen adults (Table 1) and fifteen newborn infants (Table 2).

2. The following mean figures were obtained in adults: antidiuretic activity = 14570 ± 1580 mU./gland (761 ± 58 mU./mg. dry neurohypophysial tissue); oxytocic activity = 13850 ± 1085 mU./gland (747 ± 45 mU./gm. dry neurohypophysial tissue).

3. Mean figures obtained in newborn infants were as follows: antidiuretic activity = 375 ± 40 mU./gland (166 ± 25 mU./mg. dry neurohypophysial tissue); oxytocic activity = 387 ± 20 mU./gland (150 ± 38 mU./mg. dry neurohypophysial tissue).

4. Calculated per mg. dry tissue the posterior pituitary lobes of newborn infants contained therefore only about one-fifth of the antidiuretic and oxytocic hormones found in the glands of adults. The significance of this finding is discussed with reference to the renal function of the newborn.

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A METHOD FOR TESTING THE PURITY OF COMMERCIAL SAMPLES OF T1824 WITH OBSERVATIONS ON THE AMOUNTS OF COLOURED IMPURITIES PRESENT AND THE ERRORS IN PLASMA VOLUME ESTIMATION CAUSED BY THEM

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It has been shown by a number of workers that the estimate of total red cell volume given by the dye (T1824)-haematocrit method is greater than the estimate given by reliable marked red cell methods, such as the radioactive iron (Gibson, Peacock, Seligman & Sack, 1946), the radioactive phosphorus (Hevesy, Koster, Sorensen, Warburg & Zerahn, 1944; Reeve & Veall, 1949) and the Ashby methods (Barnes, Loutit & Reeve, 1948*a*). A review of the data will be found in Reeve (1948). A possible cause of the dye-haematocrit overestimate is the presence of coloured impurities in the samples of T1824 used (Barnes, Loutit & Reeve, 1948*b*).

To determine the plasma volume a known quantity (N) of dye is injected into the circulation, and when it is distributed through the plasma the quantity (n) in unit volume of plasma is determined. Plasma volume (V) is given by the equation $V=N/n$. N and n are estimated in terms of the amount of red light absorbed by dyed plasma solutions, measurement being made with a spectrophotometer or photoelectric photometer. N is estimated from the concentration of a standard dyed plasma solution, prepared by diluting a small quantity of the dye solution with undyed plasma. This solution will therefore contain T1824 and any coloured impurities present. From the dye contents of plasma samples drawn at intervals of some minutes after the injection of dye, n is determined. If the coloured impurities present absorb significant amounts of the red light used for the photometric estimation of the dye, then their differential removal from the circulating plasma could result in erroneous estimates of plasma volume, and hence of the dye-haematocrit estimate of total red cell volume.

It has been shown that samples of T1824 may contain coloured impurities

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(Gregersen & Gibson, 1937). These workers used the spectrophotometer, an instrument neither easy to use nor generally available. As a rough test of purity Gregersen & Rawson (1943) have used the 'capillary' filter-paper test. This method may, or may not, detect coloured impurities (see, e.g., Reeve, 1948). We now describe a simple chromatographic method which may be used for the detection and rough estimation of the amounts of coloured impurities present, and a rather more complex adaptation of this method by which the amounts of the coloured impurities may be estimated accurately and their significance assessed. We report observations with both methods on five commercial samples of T1824 supplied by pharmaceutical firms, two British and three American. The simple chromatographic method should be of use in the routine testing of new samples of T 1824. The tests we report below have been made on watery solutions of dyes autoclaved for 30 min. at 15 lb. pressure, since solutions used for plasma volume estimations are treated thus.

METHODS

Simple chromatographic method. Samples of T 1824 can be adsorbed on a column of alumina. Coloured impurities and the dye itself can then be separated by elution with ammoniated aqueous ethanol mixtures of appropriate concentrations.

Apparatus. Glass tubing, diameter about 1·5 cm., length 20 cm., drawn out to a jet at one end which is lightly plugged with a small piece of cotton-wool.

Pressure bottle consisting of a stout 1000 ml. bottle connected by pressure tubing (*a*) with a sphygmomanometer bulb, (*b*) with a rubber bung fitting the head of the above glass tubing.

Reagents. Aluminium oxide, Al_2O_3, 'for chromatographic adsorption analysis according to Brockmann'; Savory and Moore Ltd., London.

Glass wool, ground fine in a mortar.

Acetic acid (1·5%); 1·5 ml. of glacial acetic acid and 98·5 ml. water.

Ammonia solution (1%); 1 ml. of NH_4OH (sp.gr. 0·88) added to 100 ml. distilled water. Because of the volatility of concentrated ammonia solutions the 1% ammonia solution was roughly titrated against HCl. 5 ml. of 1% NH_4OH was equivalent to about 8 ml. of N/10 HCl.

Absolute ethanol.

Eluting reagent A: 80 ml. of absolute ethanol and 20 ml. 1% NH_4OH.

Eluting reagent B: 20 ml. of absolute ethanol and 80 ml. 1% NH_4OH.

Preparation of column. A portion of a well-stirred mixture of alumina and distilled water is poured into the plugged glass tube. The alumina is allowed to settle and then pressed down firmly with a cork on the end of a glass rod. More of the mixture is added and the process is repeated until a uniform column of alumina about 6 cm. high is obtained. To protect the top surface a layer 3 mm. thick of ground glass-wool (added to the column as a watery suspension) is allowed to settle on the alumina. The column is washed three times with about 5 ml. of water, care being taken not to disturb the surface of the column nor to let the column run dry. The water and all succeeding solutions, unless otherwise stated, pass through the column at a steady rate of about 2 ml./min. They are forced through by air pressure applied at the head of the column by the pressure bottle. This method minimizes the escape of ammonia from the solution.

Adsorption of dye. The column is washed with 10 ml. of 1·5% acetic acid. If this is omitted some samples of alumina fail to adsorb all the added dye. About 10 mg. of dye in 10 ml. distilled water are now added carefully and the water in the solutions allowed to pass through the column slowly under atmospheric pressure. The dye is adsorbed as a narrow blue band on the top of the column and is washed with 10 ml. of distilled water.

Elution of fractions. Eluting reagent A is now added and forced through the column by air pressure. In all samples tested this separates from the dye adsorbed at the top of the column a red impurity which rapidly passes down the column. The width of band and depth of colour of the red impurity are noted. The red impurity in 10 mg. can be completely eluted and collected in about 40 ml. of solution.

When the red impurity has been removed eluting reagent B is added. This solution very rapidly brings the T 1824 fraction off the column. The T 1824 content of 10 mg. of commercial samples can be collected in about 20 ml. of reagent.

In all samples tested a small amount of a violet compound was left at the head of the column. The width of the band and intensity of its colour are noted. This compound is partly decolorized on standing. We have been unable to elute it. It is possible that it consists of more than one compound, as on occasion we have separated it into two bands.

RESULTS

Tests with the simple chromatographic method on commercial samples of T 1824. Samples of T 1824 were obtained from British Drug Houses, Ltd., and Imperial Chemical Industries, Ltd. of Great Britain, and from the Eastman Kodak Co., the National Aniline Division of the Allied Chemical and Dye Corporation, and the Warner Institute of the United States. Portions of these dissolved in distilled water were autoclaved at 15 lb. pressure for 30 min., and then fractionated on alumina columns as described above. The amounts of impurities were roughly measured from the width and intensity of the coloured bands. The results are summarized in Table 1. It can be seen that all samples of dye tested contained appreciable amounts of the red and traces of the violet impurity. The sample supplied by the Warner Institute showed the least quantity of the red impurity and that supplied by Imperial Chemical Industries the greatest. The sample supplied by the British Drug Houses showed the greatest quantity of violet impurity.

TABLE 1

Samples of T 1824 supplied by	Red impurity	Violet impurity
I.C.I.	+ + + +	+
B.D.H.	+ + +	+ +
E.K.	+ + +	+
Warner	+ +	+
N.A.D.	+ +	+

+, indicates a trace; + +, shows an easily visible quantity; + + +, a rather greater quantity than + +; + + + +, a fair amount.

The T 1824 content of plasma samples is estimated from the optical densities measured in the red in the region of 620 mμ. with the spectrophotometer or photoelectric photometer. Red impurities transmit a high proportion of red light, but violet impurities may absorb a high proportion. Appreciable amounts of red impurity are therefore unlikely to cause much error, but much smaller amounts of a violet impurity might cause error. On the basis of these rough tests samples of dye for plasma volume estimation would be selected which show a minimal quantity of violet impurity, and to be on the safe side no more than a moderate amount of the red impurity. From the results shown in Table 1 the sample supplied by British Drug Houses would be taken as insufficiently pure because of the greater proportion of the violet impurity. The quantitative tests now to be described show, however, that the amounts of impurity present in this sample are permissible.

Quantitative estimation of effects of impurities. The proportion of the total red light absorbed by the coloured impurities may be determined by comparing the optical density in the red of a known concentration of the unpurified commercial sample of dye with that of a purified sample having the same T1824 concentration. By the chromatographic method described the fraction containing T1824 can be separated from the red and violet impurities. This fraction, washed off the column with eluting reagent B, must be freed from alcohol and ammonia before its spectral properties can be compared with those of the original sample of dye. The following method was used.

The small quantity of alumina particles present was removed by filtering with suction through a sintered glass filter of porosity 4. Any dye adsorbed to the particles was freed by washing them with eluent B. The filtrate and washings were quantitatively transferred to a distillation flask. 20 mg. of sodium chloride were added to minimize adsorption of T1824 to the glass surface of the flask. The ammonia, alcohol and some of the water were then distilled off under reduced pressure.

Occasionally, in spite of the addition of sodium chloride, small quantities of T1824 became adsorbed to the distillation flask walls and were lost. By measurement it was found that the amount so adsorbed was always less than 2% of the total.

Comparison of the optical densities in the red of the unpurified and purified samples. The solvent, pH, salt content and protein content influence the absorption spectrum of solutions of T1824. Therefore these variables must be standardized. Ideally they should approximate to the conditions in serum. Two solvents have been used: (*a*) Sorensen phosphate buffer of pH 7·3 and (*b*) diluted human serum. The latter if appreciably cloudy, but not otherwise, was extracted with about one-fifth of its volume of ether to remove fat. One volume of serum was diluted with one volume of 0·9% sodium chloride solution.

Purified and unpurified dye samples were prepared for measurement as follows. The unpurified dye solution consisted of 10 mg. of the commercial sample dissolved in 100 ml. of water. The purified dye solution consisted of the T1824 fraction separated, as described above, from 10 mg. of the commercial sample and dissolved in 100 ml. of water: (1) to 5 ml. each of purified and unpurified dye solutions were added 5 ml. of Sorensen phosphate buffer of pH 7·3; (2) to 1 ml. each of purified and unpurified dye solutions were added 5 ml. of dilute serum. The optical densities of 5 mm. thicknesses of the dye solutions in phosphate buffer, and 1 cm. thicknesses of the dye solutions in serum were determined with the photoelectric photometer and red colour filters, cutting off a narrow band with its centre at 620 mμ., as described by Gibson & Evelyn

(1938). The percentage of the light absorption of the impure samples caused by the coloured impurities was then calculated from

$$\frac{D\text{ impure sample} - D\text{ pure sample}}{D\text{ impure sample}} \times 100,$$

where D = optical density. The results of a number of observations expressed thus are shown in Table 2. It will be noted that the samples supplied by the Warner Institute and Eastman Kodak have the least coloured impurities causing red light absorption, and that no sample as judged by the phosphate buffered solutions has more than 4 % of its total red light absorption caused by coloured impurities. In dilute serum solution, though one sample showed an average of 6 % of its total red light absorption to be due to coloured impurities, all the others showed 4 % or less. On the whole, the agreement shown by the estimates in phosphate and dilute serum solutions is good. Estimations on the dye contents of the dilute serum solutions are probably rather less precise, since slight variations in the light absorptions of the protein solutions themselves may influence the results. Hence results in phosphate buffer are to be preferred.

As a further check on the light absorption of the coloured impurities, the red impurity was separated from a measured amount of the Imperial Chemical Industries' sample and its red light absorption compared with that of the same amount of unpurified sample. The method used was exactly as above, except that the red impurity was quantitatively washed off the column with eluent A, collected, then purified and concentrated by vacuum distillation. The results are shown in Table 2, where it can be seen that its red light absorption in phosphate buffer of pH 7·3 is about 1 % of the total red light absorption of the unpurified sample. Hand spectroscopic examination of the watery solutions of the red impurity showed its main light absorption to lie between 600 and

TABLE 2

Mean percentages of total red light absorption of five commercial samples of T 1824 due to the presence of coloured impurities. (*a*) Measured in phosphate buffer of pH 7·3; (*b*) measured in diluted human serum

Samples of T 1824 supplied by	No. of analyses	(*a*) Mean % red light absorption of impurities in phosphate buffer	Range	(*b*) Mean % red light absorption of impurities in dilute plasma	Range
I.C.I.	4	3·9	2·5–5·8	4·1	1·6–6·8
B.D.H.	2	3·1	1·6–4·6	3·3	3·3–3·4
Warner	2	2·1	0	2·0	1·2–2·8
E.K.	2	2·0	1·7–2·3	2·5	0·8–4·2
N.A.D.	3	2·8	2·1–3·5	5·9	4·7–7·4

Mean percentage of total red light absorption of the sample of T 1824 supplied by I.C.I. due to its contained red impurity, measured in phosphate buffer of pH 7·3

Samples of T 1824 supplied by	No. of analyses	(*a*) Mean % red light absorption of impurities in phosphate buffer	Range	(*b*) Mean % red light absorption of impurities in dilute plasma	Range
I.C.I.	3	1·1	1·0–1·2	—	—

520 mμ., that is in the orange and green. The light absorptions of the red impurity and the unpurified dye sample were therefore measured in the green with the photoelectric photometer with a colour filter transmitting a band between 520 and 560 mμ. In this region, in both phosphate buffer and in plasma, the red impurity has a light absorption of about 10% of the total of that of the unpurified dye.

One observation was made on the Imperial Chemical Industries' sample to determine if prolonged autoclaving altered the relative amounts of T 1824 and its impurities. Portions of the same solutions in distilled water were autoclaved for 30 and 90 min. at 15 lb. pressure. The percentages of total red light absorbed by impurities were respectively 3·8 and 3·5%. Thus prolonged autoclaving does not increase the amounts of coloured impurities.

DISCUSSION

The results presented show that in the commercial samples of T 1824 tested between 2 and 4% of the total light absorption in the red was due to coloured substances other than T 1824. If 1% be allowed for the average losses in the method of purification then no sample contained impurities absorbing more than 3% of its total red light absorption. It is possible, but unlikely, that the fraction containing T 1824 contained other coloured impurities. This we have not completely excluded, but in many experiments with a variety of eluents, when we were searching for the best conditions for fractionating the dye samples on the column, we found no evidence that the T 1824 fraction contained other coloured compounds. The colour of the red impurity is rather similar to that of the alkaline form of T 1824, but in acid solution the two show quite different colours.

The amounts of coloured impurities found in the samples tested are too small, even if after injection the impurities are very rapidly removed from the circulation, to cause significant errors in plasma volume estimates and hence significant overestimates of total red cell volume. With less pure samples, however, error might be caused. If the purity of a sample is in doubt it is wise to test it by the simple method described. If much red, violet or other coloured impurity is found, the sample should either be discarded for a better or purified. For purifying, the impure sample may be fractionated on an alumina column as described here, the T 1824 collected, filtered and either concentrated by vacuum distillation, or precipitated by salting out.

Care should be taken in the choice of colour filters for the measurement of T 1824 concentration. Our observations show that the red impurity has a greater absorption in the green and orange than in the red. Filters and spectrophotometer slits should be used transmitting narrow bands centred at about 620 mμ. If orange or green filters are used rapid removal of the red impurity might cause significant errors.

SUMMARY

1. Tests with a simple chromatographic method, by which the presence of coloured impurities may be detected and their amounts roughly estimated, are described on five commercial samples of T1824.

2. A method is described by which the T1824 fraction and a fraction containing a red impurity can be quantitatively separated from impure commercial samples and concentrated.

3. It is shown that the coloured impurities in the five commercial samples of T1824 tested absorb at most 4% of the total red light absorption of the sample. Hence the coloured impurities present cannot be responsible for the overestimate of total red cell volume given by the dye-haematocrit method.

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THE NERVOUS PATHWAY OF THE PSYCHO-GALVANIC REFLEX

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The object of this paper is to present the results of experiments to determine the nervous pathways of the so-called psycho-galvanic reflex in man and the mechanism underlying the electrical changes of this phenomenon.

Vigoroux (1879) demonstrated that different areas of the normal skin are at different electrical potential and that these potentials vary with psychological stimuli as, for example, on raising the hat! Feré (1888) found the electrical resistance of the body to be lowered under the influence of emotional states, and that the changes were correlated with alteration of limb volume. Tarchanoff (1890) observed galvanic phenomena in the skin associated with variations in nervous activity.

Prideaux (1920), in a full and excellent review of work up to 1920, emphasizes the conflict of opinion on the physiological mechanisms underlying the psycho-galvanic reflex and concludes: '(1) That the ultimate nature of the reflex was still an unsolved problem; (2) that muscular, respiratory and secretory changes may be concomitant with emotional reactions, but that none of them is exclusively responsible for the psycho-galvanic reflex; (3) that the probable cause is to be found in biochemical changes in the skin, which are brought about by nervous excitation, but there is no evidence to show what is the nature of these changes or through what channels this nervous excitation takes place.'

Gildemeister (1923) showed that the potential and the resistance changes following such stimuli as a prick, hand clap, cough, forced expiration with closed glottis, have the same time relations.

Goadby & Goadby (1936), by simultaneous optical records of the two phenomena (resistance and potential), showed that these are actually synchronous, and that the resistance response can be abolished by complete exsanguination of the limb while the potential response persists.

Experiments on sympathectomized subjects showed that the change in skin resistance results from activity of the sympathetic nervous system and depends on two factors—sweating and vasomotor activity—(Carmichael, Honeyman, Kolb & Stewart, 1941).

In human patients, changes in finger volume, as measured by finger plethysmograph, have been shown to occur following the same stimuli as give the potential and resistance responses described above, and to have the same descending pathways in the spinal cord and in the peripheral nerves (Bolton, Carmichael & Sturrup, 1936; Gilliat, Guttman & Whitteridge, 1948; Gilliat, 1948). The observations and experiments described in this paper were started in 1935 and were interrupted by the War.

METHODS

Material

(A) *Healthy.* The two authors acted alternately as subjects of experiment and all observations on the healthy were repeated on both.

(B) *Pathological.* Observations were made on patients at St Thomas's Hospital and come under two headings: (i) cases of lumbar or cervical sympathectomy before and after operation; (ii) cases of paraplegia.

During all experiments the subjects were lying comfortably in bed or on a couch completely at rest, relaxed and warm.

After the apparatus had all been set up and the electrodes attached, no observations were made until the variations in the skin resistance and potential brought about by psychic stimuli such as noise or apprehension of the subject, were observed to have ceased; then, several short runs of the recording camera motor were made to ensure that subsequent recordings would not be complicated by responses to this stimulus.

Electrical

Skin resistance. The subject was placed in one arm of a Wheatstone bridge (Post Office box type). The resistance variations were recorded by means of a reflecting galvanometer and paper camera.

Skin potential. The subject was attached to one lead of a Cambridge portable electro-cardiograph.

Paper camera. The string of the electro-cardiograph and the spot of light from the reflecting galvanometer were focussed on the slit of a Cambridge recording paper camera driven by an electric motor with special low gearing.

Electrodes consisted of 1½ in. diameter round silver plates with silver chloride electrolytically deposited on the surface. The upper surface of the electrode and the attached lead were insulated by covering with a moulded cover of dental impression composition. The lower surface was placed in contact with a fourfold thick linen pad soaked in normal saline placed on the appropriate skin surface, which had previously been cleaned with 95% alcohol. Two broad rubber straps held each electrode in position. Two pairs of electrodes were used, one pair placed on the dorsum and palm of a hand, and one pair on the dorsum and sole of a foot, each pair was connected to a double-pole double-throw switch, so that they could be easily put into either the resistance or potential recording circuits at will without disturbing the subject. By this arrangement records were obtained simultaneously of the potential response in one limb A and the resistance response in the second B. The switches were then changed and further records obtained of the resistance changes in A and the potential in B. In all subjects records were obtained of resistance and potential changes in both hands and both feet.

Calibration. (*a*) *Potential:* 1 cm. deflexion equivalent to 10 mV. (*b*) *Resistance:* a fixed resistance (1000 Ω) was thrown in series with the subject and the deflexion on the record in mm. noted.

Time marker. ⅕ sec. recorded on the paper strip margin.

Stimuli. After preliminary experiment with such stimuli as breath holding, pricking, coughing, a sudden noise, it was finally decided to use a standard faradic stimulus from an induction coil applied to the skin of one arm through electrodes fixed in place. The time and duration of stimulus was recorded on the paper, by an electric signal.

Exsanguination

Records were obtained from an exsanguinated foot as follows: The leg was elevated, massaged from toes to knee, then an Esmarch's bandage applied from the toes up to just below the knee and there fixed. The bandage was now unwrapped from the toes to just above the ankle. If no arterial pulsation could be detected in the foot the electrodes were applied and the leg covered with a rug. At the end of the experiment it was confirmed that the foot was still cold and white without arterial pulsation.

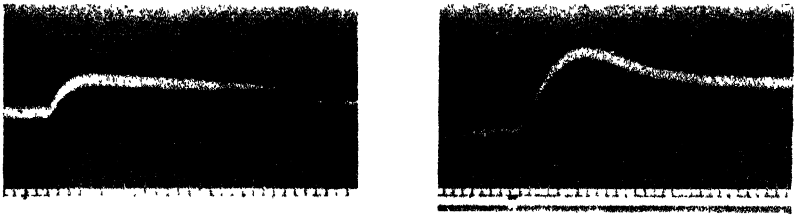
Throughout this paper *resistance response* means diminution of resistance between the two electrodes on the dorsum and sole of foot and dorsum and palm of hand respectively.

Potential response means an increase of electro-negativity in the sole of the foot with respect to the dorsum, and of the palm to the dorsum of hand.

The apparatus was adjusted so that the deflexions recorded of the responses were in the same direction, i.e. a rise indicated increase in electro-negativity or lowering of resistance in the sole or palm.

RESULTS

Exsanguination. We have previously shown that the potential and resistance responses are synchronous, the optical records showing the same latent period, the same rapid initial rise followed by a slower prolonged fall. An example of this is given in Fig. 1*a*.



a *b*

Fig. 1. Effect of exsanguination on responses in foot. White curve = potential response. Black curve = resistance response. Time marker ⅕ sec. Signal = electrical stimulus to skin of arm. *a*, responses before; *b*, responses after application of Esmarch's bandage to foot and leg, producing complete exsanguination.

Fig. 2*b* shows the abolition of the resistance response as the result of complete exsanguination of the limb with the Esmarch's bandage as described. This abolition only occurred when exsanguination of the limb was complete. In every experiment the potential response in the exsanguinated limb remained active. On removing the bandage and allowing blood to return to the limb the resistance response slowly returned. Five complete experiments were made on the two subjects: in all these the results were identical.

Sympathectomy. Records were obtained before and after six operations for cervical sympathectomy performed for Raynaud's disease in four patients. Two cases of lumbar sympathectomy were similarly investigated, in one of which records were obtained after operation only (see Fig. 2 and Table 1). These

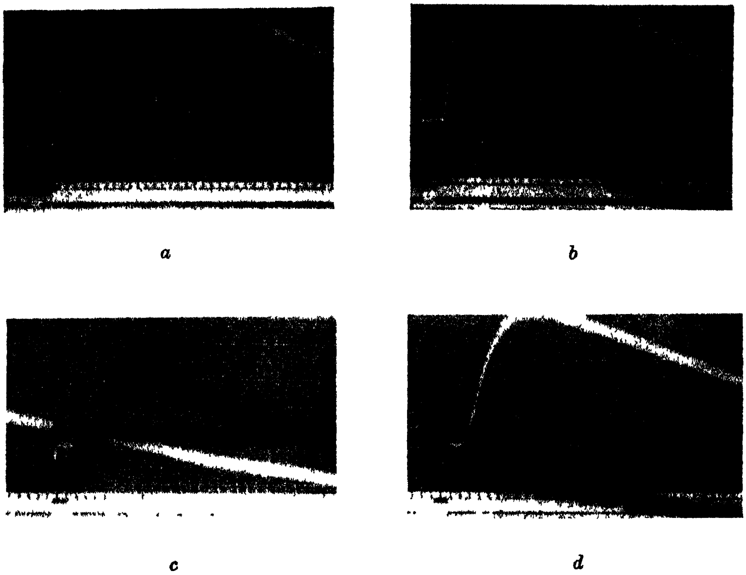


Fig. 2. Effect of left lumbar sympathectomy on responses in foot. White curve = potential response. Black curve = resistance response. Time marker $\frac{1}{5}$ sec. Signal = electrical stimulus to upper arm left. *a*, before operation: potential reponse from left foot, resistance response from left hand; *b*, before operation: potential response from left hand, resistance response from left foot; c, after operation: no potential response from left foot, resistance response from left hand; *d*, after operation: potential response from left hand, no resistance response from left foot.

TABLE 1

Summary of results obtained before and after sympathectomy,
R = resistance response, P = potential response

Case no.	Operation side	Responses: Before operation		Responses: After operation	
		Cervical sympathectomy			
1	Left hand	$R = +$	$P = +$	$R =$ Trace	$P =$ Nil
	Right hand	$R = +$	$P = +$	$R =$ Nil	$P =$ Nil
2	Left hand	$R = +$	$P = +$	$R =$ Nil	$P =$ Nil
	Right hand	$R = +$	$P = +$	$R = +$	$P =$ Trace
3	Right hand	$R = +$	$P = +$	$R =$ Nil	$P =$ Trace
4	Left hand	$R = +$	$P = +$	$R =$ Trace	$P =$ Nil
		Lumbar sympathectomy			
5	Right foot	$R = +$	$P = +$	$R =$ Trace	$P =$ Trace
	Left foot	$R = +$	$P = +$	$R =$ Nil	$P =$ Nil
6	Right foot	$R = +$	$P = +$	$R =$ Nil	$P =$ Trace
	Left foot	$R = +$	$P = +$	$R =$ Trace	$P =$ Trace
7	After operation only		Right foot	$R =$ Nil	$P =$ Trace
			Left foot	$R = +$	$P = +$

experiments demonstrate that sympathectomy profoundly diminished the responses in the operated limbs, and in the majority of instances completely abolished both. The trace of response found in some cases after operation is to be expected as complete sympathetic denervation in a limb is notoriously difficult to attain.

Nerve block. Left internal malleolar nerve. 2 c.c. novocaine-adrenaline (1 c.c. novocaine, 1 c.c. adrenaline 1/1000) were injected around the nerve at the back of the internal malleolus; complete loss of sensation in the sole of the foot followed. As a result of this nerve block the resistance response in the sole of this foot, previously very active, was almost completely abolished. The potential response, also previously active, was also reduced to approximately zero.

Paraplegia. Complete transverse lesion at level eighth dorsal segment, as the result of sarcomatous deposits in spine, proved at laminectomy. In this patient the potential and resistance responses were normal in the completely paralysed lower limbs and equal to and synchronous with those of the unparalysed upper limbs.

DISCUSSION

Various phenomena occurring in the skin in response to psychological and other stimuli have been recorded by different workers. To these phenomena the term psycho-galvanic reflex is usually applied. These are: (1) skin resistance variations first observed by Feré (1888), first recorded by Gregor & Loewe (1912); (2) the changes in skin potential first reported by Tarchanoff (1890), and first recorded by Goadby & Goadby (1936); (3) vasoconstriction first recorded by Goetz (1935), comparable to similar phenomena in the pads of animals. The latent period and form of curve recorded by the finger plethysmograph are identical with those of the other two phenomena (Golla, 1921).

The largest electrical changes are found when the electrodes are placed on the dorsum and palm of the hand, or dorsum and sole of the foot. As has been pointed out by Forbes (1936) records obtained thus are the algebraic sum of the changes occurring under each electrode.

The changes in skin resistance take place in the superficial layers of the epidermis (Golla), and many observers have said that they are due to activity of the sweat glands. Carmichael *et al.* (1941) postulated from their observations on exsanguination that the resistance response is due partly to vasomotor changes, partly to activity of sweat glands. Our experiments reported above show that when *complete* exsanguination is established the resistance response is abolished. If exsanguination is not complete traces of resistance response remain. Variations in resistance then must be due to changes in the vascular bed.

In the exsanguinated limb the potential response remained intact and active, so that this phenomenon must occur in some structure other than the vascular bed. As the potential response is abolished by sympathectomy to the limb, and as sympathectomy also abolishes the resistance response, and the vasoconstrictor response, we postulate that the potential variations are expressions of activity at sympathetic nerve endings in blood vessels, sweat glands, etc.; and that changes in skin resistance and in finger volume are due to vasoconstriction for which the sympathetic is the motor nervous pathway: only a small part, if any, of the resistance changes can be due to sweat, as the electrodes are placed on areas where sweat-gland concentration is minimal (own observations; Kuno, 1934).

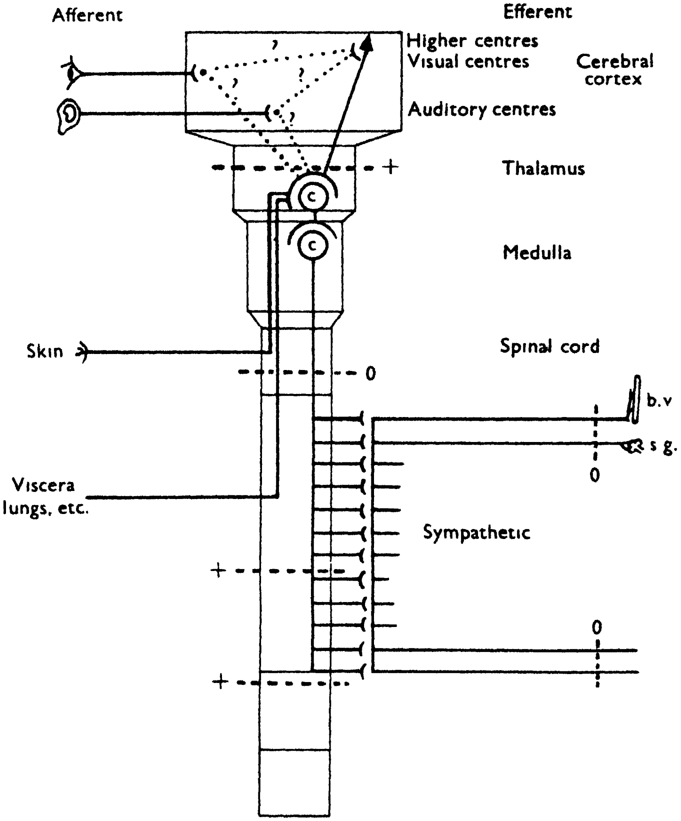


Fig. 3. Diagram of reflex pathways of psycho-galvanic reflex. The heavy broken lines show areas where division of pathways has been observed after trauma, or made by operation, with effects on the reflex and indicating reflex intact. 0, reflex abolished; *c*, hypothetical reflex centres.

Abolition of vasoconstrictor reflexes by sympathectomy has also been amply demonstrated in animals (Richter, 1929 *a* and *b*; Schwartz, 1934).

That the efferent pathway is the sympathetic is further supported by observations on patients with transection of the spinal cord at various levels, the outflow being between D1 and D8 segments of the cord.

Whatever the centre for the psycho-galvanic reflex may be, the afferent pathways must be multiple and include pain impulses whose arc goes no higher than the thalamus (Williams & Scott, 1939), afferent fibres from respiratory organs as shown by the response to forced inspiration (this paper and Gilliat *et al.* 1948), and excitation from higher cortical centres as shown by the responses to apprehension and other psychological stimuli.

The psycho-galvanic arc then consists of many afferent pathways, a centre possibly situated at the level of the thalamus or lower, and efferent pathways in the sympathetic nerves.

The end phenomena occur in the skin, and comprise variations in skin potential, in skin resistance, in the condition of the vascular bed, and activity of sweat glands.

A diagram of these pathways, and the effects of section of the nervous system at various levels is given in Fig. 3.

The reflex would more aptly be described as psycho-motor; it is probably primitive in origin and associated with preparation for activity and self-protection.

SUMMARY

1. Simultaneous records of the potential and resistance responses of the psycho-galvanic (psycho-motor) reflex were made in human beings.

2. Such records in seven patients before and after operation show that sympathectomy abolishes both the responses in the operated limb.

3. Complete exsanguination of a limb abolishes the resistance response, whilst leaving the potential response intact.

4. In a case with complete transection of the spinal cord at the level of D8, the responses in all four limbs were normal.

5. The afferent and efferent pathways of the psycho-galvanic (psycho-motor) reflex are discussed with review of the literature.

6. It is postulated that the basis of the resistance response is a change in the vascular bed, and that the basis of the potential response is activity at sympathetic nerve endings.

Our thanks are due to the physicians and surgeons of St Thomas's Hospital, London, for permission to investigate their cases, and particularly to Mr R. H. Boggon who performed the sympathectomies.

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MULTI-SENSITIZATION OF THE GUINEA-PIG UTERUS BY SERUM PROTEINS

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It has been shown (Winter, 1944*a*), using horse-serum proteins, that there is no reaction to albumin in the isolated uterus of the guinea-pig which has been sensitized to serum; nor was any response obtained to albumin when the animals had received a preliminary injection of a mixture of albumin and globulin ('Synthetic serum'), nor when the injection of albumin was made some weeks after the globulin (G-A animals). When, in separated injections of the proteins, the order of injection in the last series was reversed and the albumin was given some weeks before the globulin (A-G animals), sensitiveness to both proteins appeared in the isolated uteri, and was in nearly all cases discharged by either antigen when placed in the bath (Winter, 1945*a*). In the present work horse-serum albumin and human-serum globulin have been used. The object of the experiments was to discover whether the globulin had the same action in preventing albumin sensitiveness from appearing in the uterine muscle, as when the sensitizing proteins were homologous: also, should sensitiveness to albumin and globulin appear in the uteri, to test whether the sensitiveness to both proteins could be discharged by either of them.

METHODS

Virgin guinea-pigs were used, obtained from the Field Station of the Agricultural Research Council, and weighing about 200 g. at the time of the first sensitizing injection. The method of sensitization of the animals was, in general, the same as that described in an earlier paper (Winter, 1944*a*), except that human-serum globulin and horse-serum albumin were used. Sensitization by synthetic serum was effected by injecting into each animal a freshly prepared mixture of 2 mg. globulin and 2 mg. albumin in 0·2 c.c. normal saline: the uterine horns were tested 35–37 days later. The G–A series was sensitized by 2 mg. globulin and, 18 days later, by 2 mg. albumin: the animals were killed after a further period of 32–35 days. Albumin was injected into the A–G animals, followed by globulin 31 days later: the animals were examined 18–21 days after the sensitization by globulin. The albumin used was the same sample as served for experiments previously described (Winter, 1945*b*). Human-serum globulin was prepared by the method used for horse globulin (Winter, 1944*a*); one sample was used throughout.

On each occasion when a test of the uterine muscle was to be made *in vitro*, one horn was removed under ether anaesthesia, divided into two approximately equal parts, and each part was set up in a separate, similar, muscle bath. The test solutions of albumin and globulin, 2 mg./c.c., were freshly made each day: 0·5 c.c. was used on each occasion, no advantage having been found in using a larger amount of antigen (Winter, 1945*a*). Since tests were being made on the uteri of animals which had received sensitizing injections of albumin and globulin, and it was possible that sensitiveness to both proteins might have been established in the uteri, the procedure was as follows. Albumin was added to the bath containing one half horn; if there was a response, the muscle was allowed time to relax fully, and then a further 1 mg. albumin was added: there was never any response to such a second addition of the same antigen: after changing the Ringer 1 mg. globulin was added. To the other half horn, globulin was added, followed by globulin; then, after changing the Ringer, albumin was placed in the bath: if the first addition of globulin caused a response, no further reaction occurred when the second mg. of globulin was added. After removal of the first uterine horns for division and testing *in vitro*, injections of antigen were made into a mesenteric vein; the injections were graded, in the endeavour to avoid shock. Subsequently, the animals were killed by a blow on the head, and the remaining uterine horn was removed, divided, and tested in the baths.

RESULTS

Globulin-albumin sensitization (*globulin followed by albumin*). Eighteen animals were used. On testing the first uterine horns 50–53 days after the globulin was injected into the animals, five showed no sensitiveness to globulin nor to albumin. Two of these insensitive animals, however, died in acute anaphylactic shock 5 min. after an injection of human globulin into the portal blood, and thus reacted in a manner which had been noted in the case of animals sensitized only by horse-serum proteins (Winter, 1944*b*). The uteri of the remaining thirteen animals were all sensitive to globulin, none to albumin. This series thus agreed with the G-A series horse globulin followed by horse albumin, in that the introduction of globulin antibody into the cells rendered the sensitization by albumin apparently without effect, and the muscle was sensitive to globulin, not to albumin, when tested with these antigens in the bath.

Synthetic serum sensitization (*globulin and albumin together*). Twenty-seven animals were used. The first uterine horns of two were completely insensitive to either protein; fourteen were sensitive to globulin alone. Sensitiveness to albumin and to globulin appeared in eleven animals, a result which had not been obtained in the corresponding experiments with horse-serum proteins, and these guinea-pigs were available for studying the power of human globulin to discharge the sensitiveness to horse albumin, and the converse. It should be pointed out that it was not possible to reach a decision in every experiment in which one half of the first uterine horn showed sensitiveness to globulin, and the other half reacted when albumin was placed in the bath. Occasionally the first introduction of antigen caused such a prolonged contraction of the muscle that the preparation eventually became unreliable for further test. Of the eleven uteri showing dual sensitiveness in the synthetic serum series, only one reacted to albumin after globulin, while in ten out of the eleven there was a response to globulin after albumin. In more than half the animals sensitized by synthetic serum, human globulin resembled horse globulin in preventing sensitiveness to albumin appearing in the uterine muscle; when, however, dual sensitiveness did appear, the globulin could discharge albumin sensitiveness, while the converse occurred in only one instance.

Desensitization *in vivo* followed the same course as that *in vitro*. Six of the eleven doubly sensitive animals received injections of globulin into the portal blood after removal of the first uterine horns. Shock occurred in one animal, but recovery took place. In no instance was the second horn sensitive to albumin when the animals were killed 3–4 hr. later. The remaining five guinea-pigs received injections of albumin: two died in acute shock but the three others were successfully desensitized to albumin, though not to globulin, the uteri still remaining sensitive to this protein when placed in the bath.

Albumin-globulin sensitization (albumin followed by globulin). Thirty-eight guinea-pigs were sensitized, and examined 49–52 days after the injection of albumin. Twenty-two animals were sensitive to both, and nine to neither protein; seven were sensitive to globulin alone. The first uterine horns of ten of the twenty-two animals which showed dual sensitiveness, reacted to globulin after albumin had been placed in the bath. When the antigens were added in the reverse order to the other half horns, two showed very prolonged responses to globulin and were discarded: of the remaining twenty, only two had retained sensitiveness to albumin. Comparing these results with those obtained from the animals sensitized by synthetic serum, it is seen that the longer the albumin antibody exists within the cell, the more capable it is of discharging sensitiveness to globulin when the reaction takes place between albumin and the albumin antibody. Globulin antibody was present in the cells for the same period in the A-G and synthetic serum-sensitized animals, and no difference was observed in the two series when albumin followed globulin in the bath.

The doubly sensitive A-G animals were desensitized *in vivo* by globulin and albumin respectively, after the tests of the first uterine horns, with results which were similar to those obtained by desensitization of the muscle *in vitro*. Globulin was injected into twelve animals; in only one was the second uterine horn sensitive to albumin. Ten guinea-pigs received injections of albumin into the portal blood: three died in acute shock; of the remaining seven animals, two showed responses to globulin when the second horns were tested.

DISCUSSION

The results obtained from the G-A animals in the present work are identical, as regards the uterine muscle, with those from animals sensitized by horse-serum globulin and horse-serum albumin; in both cases the globulin was able to prevent the albumin from forming an antibody which could show itself by response to albumin placed in the bath. It has been suggested that though only globulin sensitiveness appears in the uteri of these animals, the antibody is not identical with globulin antibody, and albumin has taken some part in its formation: more recent work (Winter, 1945*b*), though not showing this view to be wrong, has rendered it less likely.

The apparent identity between the present and earlier results ceases with those obtained from the G-A sensitized animals. The uteri of guinea-pigs sensitized by synthetic serum, in which the albumin and globulin were obtained from different species, were in many cases responsive to albumin *in vitro*; in the earlier experiments, when the proteins were both from horse serum, no such response to albumin was discovered, the globulin always being able to prevent sensitiveness to albumin from appearing in the muscle. A considerable number of animals were sensitive only to globulin, and, as in the case of the G-A animals, the question of the identity of the antibody with globulin antibody must be left undecided. The antibody in the uterine muscle of the doubly sensitive S-S animals shows some resemblance to that in the muscle of the doubly sensitive A-G animals, both those sensitized by horse-serum albumin and horse-serum globulin, and those described in the present paper in which the globulin was of human origin. The resemblance lies in the ability of globulin to discharge sensitiveness to albumin *in vitro*; there is no significant difference in the three series (see Winter (1945*a*) for the A-G animals sensitized by horse-serum proteins). The three series differ markedly, however, as regards the capacity of albumin to discharge sensitiveness to globulin: when both proteins were derived from the horse, albumin as often discharged globulin sensitiveness as did globulin the reactivity to albumin. In the uteri of the eleven doubly sensitive S-S animals, albumin entirely failed to discharge sensitiveness to human globulin in ten of the experiments: it was more successful, however, in the A-G series horse albumin-human globulin, where the albumin antibody had been established in the cells for a longer period; in these animals the sensitiveness to globulin was discharged by albumin in more than half the experiments.

A point of interest arises from consideration of the large number of animals in the A-G series described in the present paper, which failed to become sensitized to albumin. This protein was the first to be injected into the animals, and only when albumin antibody had had ample time to become established in the cells was the sensitizing injection of globulin given. Of the thirty-eight guinea-pigs which received the two preliminary injections, the uteri of sixteen, or 42 %, showed no sensitiveness to albumin *in vitro*. It is possible that albumin is a less successful antigen than globulin in the guinea-pig, and it might be argued that such a large number of animals insensitive to albumin in the series under consideration, is not abnormal. My results do not support this view: taking figures derived from guinea-pigs comparable in weight and all obtained from the same source as those in the present work, and sensitized only by horse globulin or by horse albumin, there was no difference between the results of sensitization. Of the 118 globulin-sensitized and 85 albumin-sensitized animals, 29 and 21 respectively, or 24%, were insensitive. In the case of the albumin-sensitized animals, the extremes were 16 and 29% insensitive, in the different batches. The high value of 42% insensitive obtained in the A-G series horse albumin-human globulin, does at least raise the question whether the heterologous globulin is able to displace albumin antibody from the cells; it is possible that there was, initially, a larger number of animals sensitive to albumin in this series, but that when they were examined some 3 weeks after the sensitizing injection of human globulin had been given, the latter protein had, in the course of antibody formation, displaced albumin antibody from the cells of some of the uteri.

SUMMARY

1. A study has been made of the sensitizing action on guinea-pigs of human-serum globulin and horse-serum albumin.
2. When the sensitizing injection of globulin is given some weeks before the albumin, sensitiveness to the latter protein does not appear in the uteri.
3. When equal amounts of the sensitizing proteins are mixed and given as a single sensitizing injection, and when the globulin is injected some weeks after the albumin, the animals may become sensitized to both proteins.
4. The desensitizing action of the two proteins has been studied in animals which were sensitive to both antigens.

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THE LIBERATION OF HISTAMINE BY CERTAIN ORGANIC BASES

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Histamine is liberated from mammalian tissues by a variety of agents, most of which are of a complex nature chemically. They include antigens (in sensitized animals), peptone, trypsin, lysolecithin, animal venoms and certain bacterial toxins (for references see Feldberg, 1941). In addition, several basic drugs of relatively simple structure have been reported to liberate histamine. The release of histamine from skeletal muscle by curare alkaloids (Alam, Anrep, Barsoum, Talaat & Wieninger, 1939; Schild & Gregory, 1947) and by strychnine (Schild & Gregory, 1947) is well established; and that adrenaline has a similar action has been reported by Eichler & Barfuss (1940) and by Staub (1946).

In the course of some pharmacological tests on licheniformin, an antibiotic base extracted from *Bacillus licheniformis* by Callow, Glover, Hart & Hills (1947), we found that the characteristic lowering of the cat's blood pressure produced by the substance was due to the liberation of histamine. Further investigation showed that many other organic bases produced a depressor effect of the same kind, and for the same reason.

A preliminary report of the results has already been made (MacIntosh & Paton, 1947).

METHODS

Most of our experiments were carried out on cats or dogs. Cats were usually anaesthetized with chloralose; a few were anaesthetized with ether, or were decerebrated under ether. Dogs were anaesthetized with sodium barbitone, sodium phenobarbitone or a chloralose-urethane (1:10) mixture. Blood pressure was recorded in the usual way from a carotid artery, the anticoagulant fluid being either saturated Na_2SO_4 solution, or more usually 0·9 % NaCl containing heparin. Blood samples for pharmacological tests were taken only in experiments in which no blood-pressure record was made; they were withdrawn from a carotid artery through a steel cannula filled with 1 % sodium heparin solution, into a syringe containing 0·05 c.c. of the same solution for each c.c. of blood. Plasma for pharmacological tests was obtained by centrifuging such blood samples without delay. We found that normal plasma so collected had no depressor activity when reinjected. Plasma samples and extracts were tested on the isolated ileum of the guinea-pig, suspended in Locke's solution containing 0·004 % $MgCl_2$; the volume of the bath was 20 c.c. For some tests, atropine sulphate (4×10^{-7}) or Neoantergan maleate ('Anthisan', May & Baker: 10^{-9}) was added to the fluid in the bath.

Other procedures are described in the text. Doses and concentrations of histamine are stated in terms of the base.

RESULTS

Experiments with licheniformin

Our first experiments were made with preparations of 'licheniformin hydrochloride' supplied by Dr R. K. Callow. The material so designated appears to be a mixture of basic compounds of predominantly polypeptide nature (Callow & Work, 1948). The preparations used by us were probably not of identical composition, but they were qualitatively similar in action.

The delayed depressor effect. In a cat anaesthetized with chloralose, the first effect on the arterial pressure of a small intravenous dose (e.g. 1–2 mg./kg.) of licheniformin hydrochloride is a rapid fall which closely resembles that produced, for instance, by a small dose of histamine (Fig. 1). But whereas the depressor effect of substances such as histamine or acetylcholine becomes evident a few seconds after the injection, that of licheniformin always has a longer latency, usually about 20–25 sec. Once it has begun, however, the fall in blood pressure with licheniformin may be just as rapid as with histamine. The depressor effect of such a dose of licheniformin is sometimes transient, but more often the initial level of blood pressure is not regained for several minutes. With a somewhat larger dose (e.g. 4 mg./kg.) the fall of arterial pressure is more severe and prolonged, and a yet higher dose (e.g. 10 mg./kg.) produces lasting circulatory shock; but the characteristic delay in the appearance of the depressor effect is still observed whatever the amount injected.

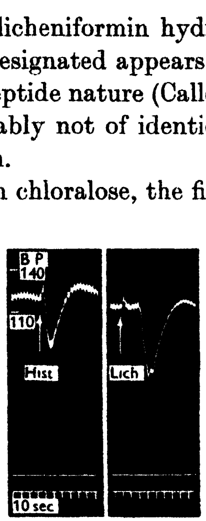


Fig. 1. Cat, chloralose: blood pressure. Hist., injection of 0·2 μg./kg. of histamine. Lich., injection of 0·95 mg./kg. of licheniformin hydrochloride.

In the cat anaesthetized with ether, or decerebrated, licheniformin sometimes causes a small immediate fall of blood pressure, which is followed by, or merges into, a steeper fall occurring after the usual latency.

The depressor action of licheniformin is unaffected by atropinization or by section of the vagi. The heart rate is initially unaffected, though cardio-acceleration, presumably reflex or due to circulating adrenaline, may be seen during the recovery phase; and the heart continues to beat strongly even when the blood pressure has been reduced to a low level by a large dose of licheniformin. That the site of the depressor action is predominantly the peripheral vessels is readily shown. The fall of arterial pressure is accompanied by an increase in limb volume (Fig. 2); and when the drug is injected alternately into the right and left auricle, the fall in blood pressure is somewhat greater in the latter case, and begins 2–3 sec. earlier (Fig. 3). In the cat the depressor effect of a small dose of licheniformin is reproducible only if the injections are spaced 10 min. or more apart. With more frequent injections, the second usually evokes a bigger response than the first, and a dose which was initially below threshold may produce a large effect when repeated (Fig. 4). Even when the injections are timed so as to avoid this phenomenon the slope of the dose-response curve is found to be peculiarly steep. Thus, in one experiment, 1·0 mg. of licheniformin had no perceptible effect on the arterial pressure, but 1·5 mg. produced a fall of 45 mm. Hg. In animals whose blood pressure has been much reduced as the result of previous treatment with licheniformin, the form of the depressor effect is somewhat modified, the fall being smaller and less abrupt, and the latency longer.

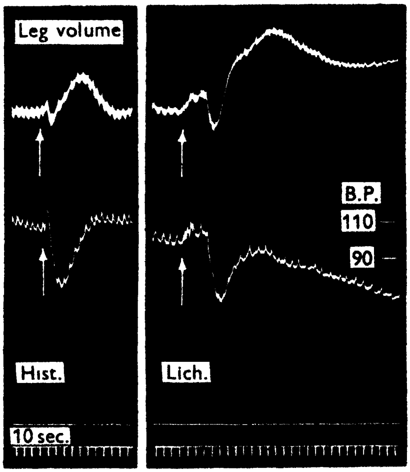


Fig. 2. Cat, chloralose: hindlimb volume and blood pressure. Hist., injection of 0·8 μg./kg. histamine. Lich., injection of 4 mg./kg. licheniformin hydrochloride.

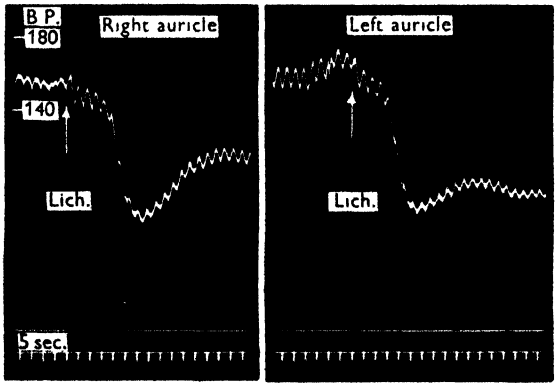


Fig. 3. Cat, chloralose, chest opened, artificial respiration. Blood pressure. Injection of 1·1 mg./kg. of licheniformin hydrochloride into right and left auricle respectively.

The mechanism of the depressor effect. Its long latency and other peculiarities suggested that the depressor effect might be due, not to a direct action of licheniformin itself on the vessels, but to the formation or liberation, as a result of its presence in the blood, of some vasodilator substance. We therefore tested the effect of incubating licheniformin with heparinized cat's blood. The reinjection of such blood, however, produced only a delayed depressor effect, such as is produced by licheniformin itself. Contact between the drug and blood thus appeared to give rise to no new depressor substance, but it was possible that contact between the drug and the tissues might do so; the next step, therefore, was to test for depressor activity the blood of a cat which had received licheniformin. For this purpose arterial blood was withdrawn and heparinized (the heparin was free from depressor substances), and immediately injected into the vein of a second cat whose blood pressure was being recorded. The blood pressure of the donor cat was not recorded, lest the results should be complicated by the entry of anticoagulant fluid into the circulation. This experiment, in which the dose was 10 mg./kg. of licheniformin hydrochloride, gave a clear result (Fig. 5). Two c.c. of blood, withdrawn from the donor before the injection, had no effect on the blood pressure of the recipient, but the same volume of blood withdrawn 1 min. after the injection produced a sharp transient lowering of the recipient's blood pressure. This depressor effect had only a brief latency (6–8 sec.) and was, therefore, not due to licheniformin itself, which in any case could hardly have been present in significant quantity. Later blood samples from the donor cat showed diminishing depressor activity.

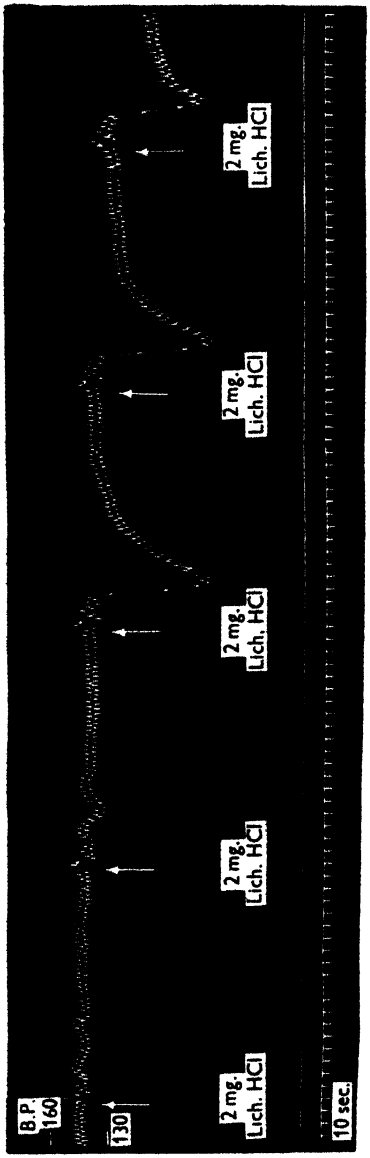


Fig. 4. Cat, chloralose: blood pressure. At 3 min. intervals, injection of 0·65 mg./kg. of licheniformin hydrochloride.

Identification of the depressor substance. Further tests were carried out with heparinized plasma instead of whole blood, which produces non-specific pressor

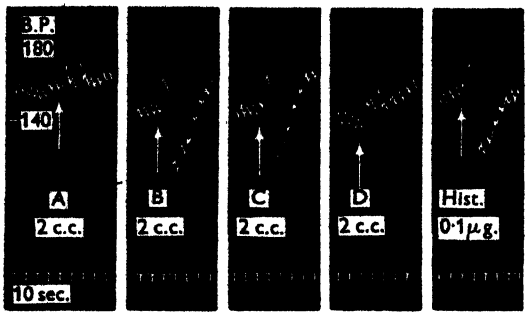


Fig. 5. Cat, chloralose: blood pressure. Injection of heparinized arterial blood samples from a second cat: A, sample obtained before injection of licheniformin hydrochloride (10 mg./kg.); B, C and D, samples obtained 1, 13 and 35 min. after the injection. Hist., histamine.

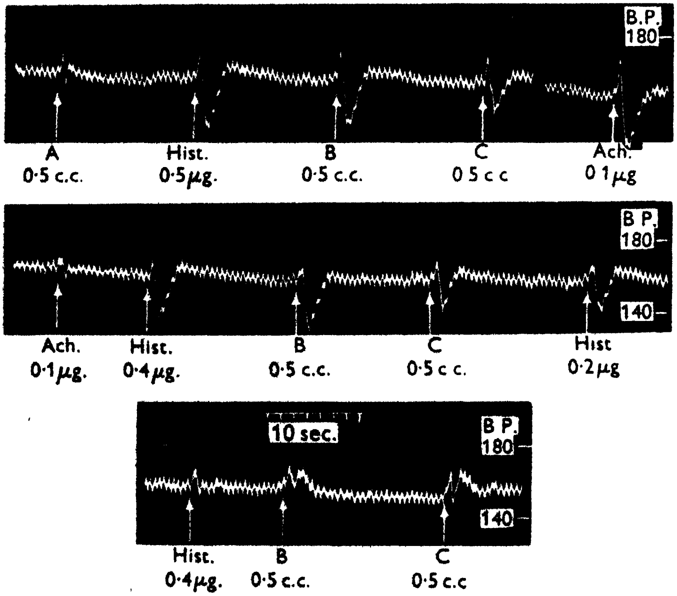


Fig. 6. Cat, chloralose: blood pressure. Injection of heparinized arterial plasma samples from a second cat. A, sample obtained before injection of licheniformin hydrochloride (30 mg./kg.); B, C, samples obtained 1 and 35 min. after the injection. Hist., histamine. Ach., acetylcholine chloride. Between first and second tracings, injection of 0·8 mg./kg. of atropine sulphate; between second and third tracings, injection of 0·8 mg./kg. of Neoantergan maleate.

effects on intravenous injection, and gives rise to inconvenient foaming when tested in the isolated-organ bath. Plasma from cats which had received licheniformin produced effects on the blood pressure strikingly like those of histamine, and when the concentration of the depressor material was assayed in terms of histamine at various levels of dosage the same estimate was obtained. The depressor effect of such plasma was unchanged by previous administration of atropine in an amount sufficient to annul the effect of an equidepressor dose of acetylcholine, but it was abolished by an amount of Neoantergan just sufficient to abolish the effect of an equidepressor dose of histamine. Bovet and his co-workers (Bovet, Horclois & Walthert, 1944; Bovet, Horclois & Fournel, 1944) have shown that Neoantergan is a fairly specific antagonist to most of the effects of histamine, including its depressor action. Fig. 6 is the record of a typical experiment.

In addition to its depressor activity, plasma withdrawn from a cat which had received licheniformin produced a contraction of the isolated guinea-pig's ileum, indistinguishable from that produced by histamine. (Licheniformin itself in concentrations as high as 10^{-4} had no effect on the isolated gut, and did not modify its response to histamine.) It was usually possible to express the activity of the plasma in terms of the equivalent concentration of histamine, and the values then obtained agreed with those found in a parallel assay on the cat's blood pressure, within the error of the two methods. In some experiments plasma obtained before the injection of licheniformin either produced a slow contraction of the gut muscle, or modified its sensitivity to histamine, so that the quantitative assay failed; but it was always clear that the injection of licheniformin had greatly increased the histamine-like activity of the plasma. The addition to the bath of atropine sulphate to give a concentration of 4×10^{-7}, which abolished the effect of an equipotent dose of acetylcholine, slightly reduced the response to post-licheniformin plasma, and in about the same degree the response to histamine. Neoantergan (10^{-9}) abolished the response to post-licheniformin plasma and to histamine, but not to other stimulants of smooth muscle (acetylcholine, K^+, Ba^{++}). The pre-treatment of the gut with excess of histamine (Barsoum & Gaddum, 1935) had a similar effect.

Finally, extracts of plasma were made by Code's (1937) modification of the method of Barsoum & Gaddum (1935), involving the destruction through acid hydrolysis of most of the substances acting like histamine on the blood pressure and on the isolated intestine which are likely to be present in tissue extracts. The extracts were assayed against histamine on these two test objects, atropine being present in each case. The results agreed with each other, and with those obtained for the fresh plasma. Fig. 7 and Table 1 give the result of such an experiment.

Thus, soon after the administration of licheniformin to a cat its plasma is found to contain a depressor substance which is not licheniformin itself, but which resembles histamine not only in its depressor effect, but also in its action on

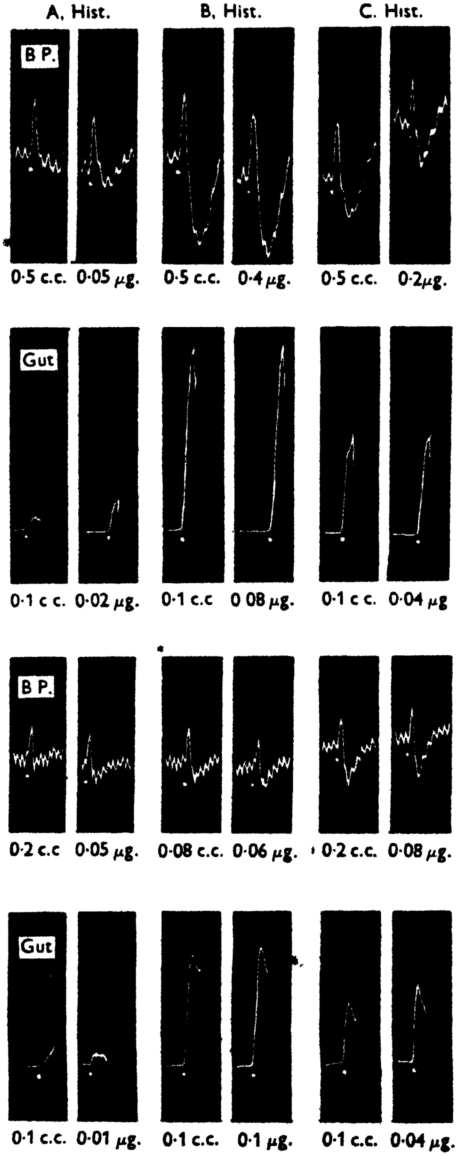


Fig. 7. Assay of samples of heparinized cat's plasma on cat's blood pressure and guinea-pig's intestine. A, plasma obtained before injection of licheniformin hydrochloride 30 mg./kg. B and C, samples obtained 1 and 35 min. after the injection. Hist., histamine. The upper half of the figure compares the effects of histamine and the untreated plasma; the lower half compares the effects of histamine and extracts of the plasma made by Code's method. Volumes are expressed in terms of the original plasma.

intestinal smooth muscle, in its behaviour in the presence of atropine, Neoantergan, and excess of histamine, and in its resistance to acid inactivation. This resemblance, and the quantitative agreement among the results of all the tests in which post-licheniformin plasma was compared with histamine, seem to us sufficient evidence for the conclusion that the depressor substance is histamine itself, and that no other substance contributes in any important degree to the depressor activity. As there is no reason to think that the cat can convert licheniformin into histamine, it must be supposed that histamine is discharged from the tissues under the influence of licheniformin. We have some direct evidence that this occurs; in the one experiment of this type that we have made, the histamine content of the skin of a cat's leg fell from 32 to 20 μg./g., after the injection of a large dose of licheniformin.

TABLE 1. Histamine equivalent (μg./c.c.) of plasma from a cat treated with licheniformin hydrochloride (30 mg./kg.)

	Before licheniformin	1 min. after licheniformin	35 min. after licheniformin
Fresh plasma, tested on blood pressure	<0·10	0·80	0·40
Fresh plasma, tested on gut	<0·10	0·80	0·42
Plasma extracts, tested on blood pressure	<0·20	0·75	0·40
Plasma extracts, tested on gut	<0·10	0·90	0·35

Experiments with amines, amidines, guanidines and isothioureas

Since licheniformin concentrates gave the Sakaguchi test for guanidine derivatives (Callow *et al.* 1947), we examined a number of bases containing the guanidine group or a related radical. (Unless otherwise noted, compounds were tested in the form of their hydrochlorides or dihydrochlorides.) Some of these were found to act on the chloralosed cat in the same way as licheniformin, the blood pressure beginning to fall steeply some 20 sec. after an intravenous injection, with no immediate change of the heart rate. The active compounds tested varied considerably in effectiveness; some were more and some less active than licheniformin. In a few cases there appeared to be some minor variations in the abruptness and the duration of the blood-pressure fall, but more often the blood-pressure records were indistinguishable from those made with licheniformin. Compounds which produced the delayed depressor effect showed two other phenomena which we have described for licheniformin: the increased response to the second of two small doses, and the large increase of effect with a small increase of dose.

Of the compounds which acted like licheniformin on the blood pressure, the simplest were aliphatic *diamines* homologous with ethylene diamine (Fig. 8). Trimethylene diamine and tetramethylene diamine (putrescine) were inactive. Cadaverine (pentamethylene diamine) lowered the blood pressure, as Barger & Dale (1910) found, but in this case the effect began within a few seconds of the injection. The C_6 base, however, produced a typical delayed effect; it was about one-quarter as active as licheniformin. Steadily increasing activity was shown by the members of the series with 7, 8, 9, 10 and 11 carbon atoms, the latter being about twice as effective as licheniformin; the C_{12} base was somewhat less active. The C_{16} base produced a mixture of pressor and depressor effects, and appears to combine the vasoactive properties of long-chain primary monoamines (Barger & Dale, 1910) with those of the shorter diamines.

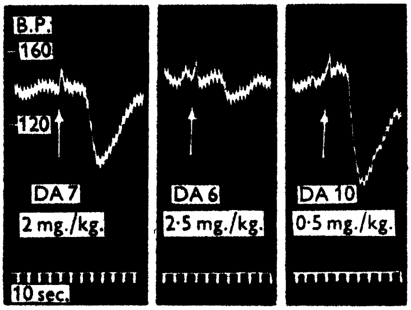


Fig. 8. Cat, chloralose: blood pressure. Delayed depressor responses to polymethylene diamines: doses refer to dihydrochlorides. DA 7, diaminoheptane; DA 6, diaminohexane; DA 10, diaminodecane. 10 min. between injections.

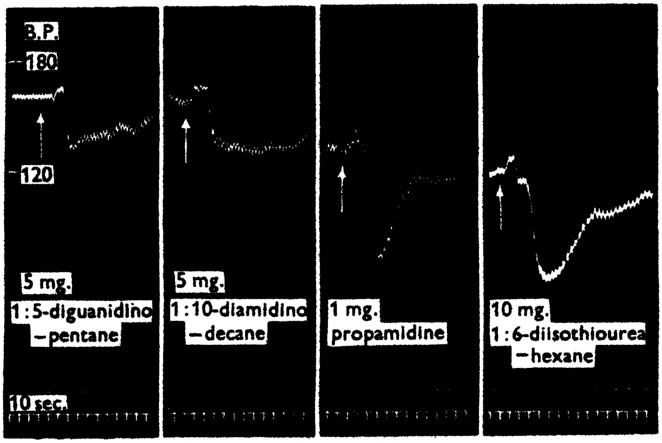


Fig. 9. Cat, chloralose 2·5 kg.: blood pressure. Delayed depressor responses to compounds with basic groups at each end of the molecule. Doses refer to dihydrochlorides.

Simple aliphatic *diamidines*, *diguanidines* and *diisothioureas*, having the basic groups placed at either end of a carbon chain, also produced the delayed depressor effect (Fig. 9); they appeared to be somewhat more active than the corresponding diamines. Two diquaternary bases, α-ω-*bis*-trimethylammonium decane and dodecane diiodide, were tested. In the doses used these lower the cat's blood pressure through their action in paralysing sympathetic ganglia; but in the fully nicotinized cat, in which such action is no longer demonstrable, a typical delayed depressor response was obtained. We have also been able, under the same conditions, to obtain a typical effect with D-tubocurarine chloride, as would be expected from the finding of Schild & Gregory (1947) that this compound can liberate histamine. We examined a number of aliphatic primary monoamines, monoamidines, monoguanidines and monoisothioureas; they produced various effects on the blood pressure, but none resembling that of licheniformin. Many compounds of this sort, indeed, are pressor agents (Fastier & Smirk, 1943, 1947).

The ability to produce the delayed depressor effect is not confined to simple aliphatic bases. The most active substances we tested, in fact, were members of the series of trypanocidal aromatic diamidines investigated by King, Lourie & Yorke (1938). Two of these, stilbamidine (4:4-diamidinostilbene) and propamidine (4:4-diamidinodiphenoxypropane; Fig. 9) were about three times as effective as licheniformin. Wien (1943), who described the depressor action of these and related substances, did not refer to its peculiar time relations, but his tracings clearly show the characteristic latent period. We also found a number of *monoamidines* derived from benzamidine which produced the delayed depressor effect; some of these compounds, whose antibacterial and antirickettsial properties have aroused interest, have other pharmacological effects resembling those of the trypanocidal diamidines (Dawes, 1945). In this series, the presence of a polar group remote from the terminal amidine radical appears to be a necessary, though not sufficient, condition for the presence of the characteristic depressor activity. Our tests, however, have been confined to those compounds which were readily available to us, and we have not so far attempted any close study of the relations between chemical structure and activity of the licheniformin type.

Some of the effective compounds, especially those with long aliphatic chains, lower surface tension at an air-water interface when present in high dilution. This surface activity did not appear to run parallel with depressor activity, nor did such highly surface-active substances as saponin, bile salts, soaps or cetyltrimethylammonium bromide produce the delayed depressor effect.

The compounds which have been tested for their ability to produce the characteristic delayed depressor effect are listed in Table 2, and the approximate threshold dose is stated for each compound which clearly produced such an effect. No such activity was detected for the other compounds listed, either because they have little or no power of liberating histamine or because they had effects of a different sort on the blood pressure which would have masked any vasodilatation due to released histamine; in the case of all these compounds, we have stated, with the sign > prefixed, the highest dose tested which certainly

TABLE 2

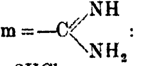
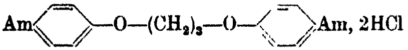
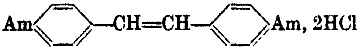
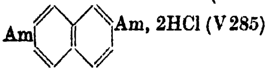
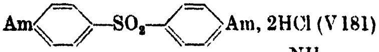
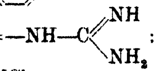
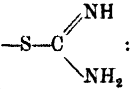
No.	Compound	Approximate threshold dose (mg./kg.)	Remarks
1	Licheniformin hydrochloride	0·3–1·0	—
	Diamines:		
2	$NH_2(CH_2)_2NH_2,2HCl$	>20	Slight depressor action of short latency
3	$NH_2(CH_2)_3NH_2$, 2HCl	>8	—
4	$NH_2(CH_2)_4NH_2$, 2HCl	>9	Slow depressor action of short latency
5	$NH_2(CH_2)_5NH_2$, 2HCl	>2	Depressor effect of short latency
6	$NH_2(CH_2)_6NH_2$, 2HCl	1·8	—
7	$NH_2(CH_2)_7NH_2$, 2HCl	1·25	—
8	$NH_2(CH_2)_8NH_2$, 2HCl	0·9	—
9	$NH_2(CH_2)_9NH_2$, 2HCl	0·8	—
10	$NH_2(CH_2)_{10}NH_2$, 2HCl	0·5	—
11	$NH_2(CH_2)_{11}NH_2$, 2HCl	0·5	—
12	$NH_2(CH_2)_{12}NH_2$, 2HCl	1·0	—
13	$NH_2(CH_2)_{16}NH_2$, 2HCl	10	Pressor action also
	Diamidines, Am =  $-C{\overset{\displaystyle NH}{\underset{\displaystyle NH_2}{\lessgtr}}}$:		
14	$Am(CH_2)_8Am$, 2HCl	1·2	—
15	$Am(CH_2)_{16}Am$, 2HCl	1·5	Pressor action also
16	 (Propamidine)	0·3	
17	 (Stilbamidine)	0·3	—
18		3	—
19		0·3	—
	Diguanidines, Guan =  $-NH-C{\overset{\displaystyle NH}{\underset{\displaystyle NH_2}{\lessgtr}}}$:		
20	$Guan(CH_2)_5Guan$, 2HCl	2·5	—
21	$Guan(CH_2)_{10}Guan$, 2HCl (Synthalin)	1	—
22	$Guan(CH_2)_{18}Guan$, 2HCl	7	Pressor action also
	Diisothioureas, ITU =  $-S-C{\overset{\displaystyle NH}{\underset{\displaystyle NH_2}{\lessgtr}}}$:		
23	$ITU(CH_2)_6ITU$, 2HBr	2·8	—
24	$ITU(CH_2)_{16}ITU$, 2HBr	2	—
	Diquaternaries:		
25	$(CH_3)_3N(CH_2)_6N(CH_3)2I$	>10	All these compounds were tested after paralysis of autonomic ganglia with nicotine tartrate or tetraethylammonium iodide
26	$(CH_3)_3N(CH_2)_{10}N(CH_3)_32I$	>4	
27	$(CH_3)_3N(CH_2)_{12}N(CH_3)_32I$	5	
28	*d*-Tubocurarine chloride	0·6	
29	*o*-Methyl-*d*-tubocurarine iodide	0·6	

TABLE 2 (*cont.*)

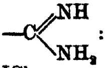
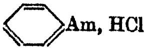
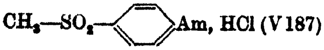
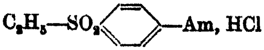
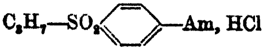
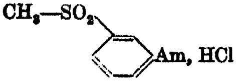
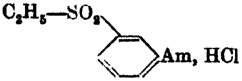
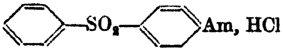
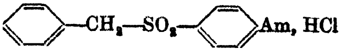
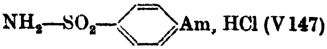
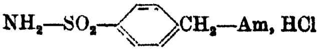
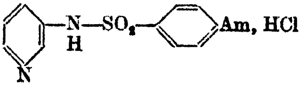
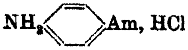
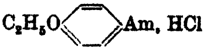
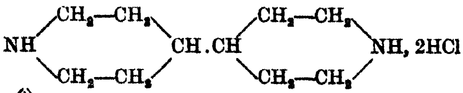
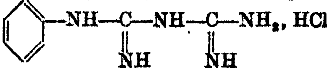
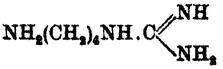
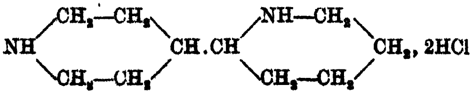
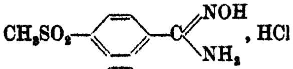
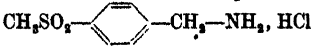
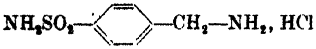
No.	Compound	Approximate threshold dose (mg./kg.)	Remarks
	Monoamidines, Am =  $-C(=NH)NH_2$:		
30	$SO_2NH_2(CH_2)_6Am$, HCl	6	—
31		>18	Feeble pressor action
32		5	
33		6	—
34		4	—
35		5	—
36		5	—
37		1	—
38		>3	—
39		9	—
40		>17	—
41		8	—
42		6	
43		6	—
	Miscellaneous:		
44		2·5	—
45		3·5	—
46	 $NH_2(CH_2)_4NH.C(=NH)NH_2$	30	—
47		>4	—

TABLE 2 (*cont.*)

No.	Compound	Approximate threshold dose (mg./kg.)	Remarks
48		>13	Cf. no. 32
49		>30	—
50	 (Marfanil)	>30	—
51	Guanidine carbonate	>18	—
52	Creatinine (Base)	>7	—
53	Nicotinamide	>4	—
54	Aneurine chloride	>40	—
55	Paludrine acetate	>4	Depressor action of short latency
56	Neoarsphenamine	>100	Depressor action of short latency
57	Procaine hydrochloride	>12	Gradual depressor action of short latency
58	Strychnine sulphate	>3	Convulsant
59	Tetramethylammonium iodide	>45	After paralysis of autonomic ganglia with nicotine
60	6-Methoxy-4-quinolylethylamine, HCl	>4	Convulsant
61	6-Methoxy-4-quinolylethylguanidine, HCl	>3	Depressor action of short latency
62	Saponin (B.D.H.)	>0·7	Depressor action of short latency
63	Sodium glycotaurocholate	>4	Depressor action of short latency
64	Cetyltrimethylammonium bromide	>4	—

failed to produce the delayed fall of blood pressure, and the nature of the blood-pressure response where one was observed.

We expected that the compounds listed here as active would act, like licheniformin, by the liberation of histamine. This expectation has been verified for two of them, propamidine and 1:8-diamino-octane, by tests identical with those already described for licheniformin. Table 3 gives the results obtained in the diamino-octane experiment, and Fig. 10 shows part of the pharmacological comparison between histamine and the plasma obtained in the propamidine experiment. Similar tests on dogs with propamidine and another substance, 1:10-diamidinodecane, have given equally clear-cut results. The experiments on dogs will be discussed in detail in a later section.

These results, together with others described below, have made us confident that all the compounds listed as active in Table 2 act on the circulation by the same mechanism. We shall, therefore, refer to them henceforth as 'histamine liberators'.

Although we do not know of any compound, other than those to which we have referred as 'histamine liberators', whose effect on the cat's blood pressure is at all similar to that of licheniformin, we do not wish to suggest that an activity of this type is in itself proof that a compound acts by liberating histamine. If, however, the compound is closely similar in structure to one of the known liberators, the presumption would be strong that its mode of action is the same.

TABLE 3. Histamine equivalent (μg./c.c.) of plasma from a cat treated with diamino-octane dihydrochloride (13 mg./kg.)

	Before diamino-octane	1 min. after diamino-octane	34 min. after diamino-octane
Fresh plasma, tested on blood pressure	<0·03	0·63	0·16
Fresh plasma, tested on gut	<0·01	0·68	>0·08 <0·20
Plasma extracts, tested on blood pressure	<0·15*	0·70*	0·30*
Plasma extracts, tested on gut	0·02	0·53	0·16

* Depressor activity not wholly abolished by Neoantergan.

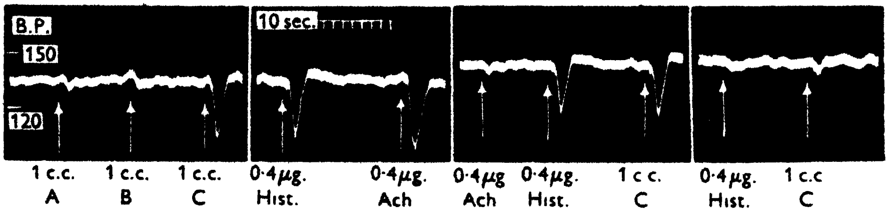


Fig. 10. Cat, chloralose: blood pressure. Effect of histamine and of heparinized plasma from a cat anaesthetized with chloralose. A and B, control plasma samples. C, plasma withdrawn 1 min. after injection of propamidine dihydrochloride 5 mg./kg. Hist., histamine. Ach., acetylcholine chloride. Between the second and third parts of the record the cat received 0·4 mg./kg. of atropine sulphate, and between the third and fourth parts of the record it received 0·4 mg./kg. of Neoantergan maleate.

Other effects of histamine liberators

The experiments so far described have dealt with the effect of histamine liberators on the blood pressure of cats. The compounds of this group have other characteristic effects which appear to depend on the release of histamine.

Intestine. It has already been mentioned that licheniformin has no action on the isolated ileum of the guinea-pig. The same was found to be true of the other histamine liberators we have tested (nos. 8, 14, 16, 20 and 23 of Table 2), when added in concentrations up to 10^{-4} to the Locke's solution surrounding the intestinal strip. As it seemed possible that the alkalinity of Locke's solution might interfere with the action of histamine liberators on the gut, the experiment was repeated with a modified Tyrode's solution adjusted to pH 7·3, but the same negative result was obtained. In concentrations of 10^{-3} or more the guinea-pig's gut responded in some experiments with a brief contraction. Wien (1943) observed that the therapeutic diamidines in similar concentrations contracted the isolated gut of the rabbit. Thus, although the guinea-pig's intestine is known to have a fairly high content of histamine, this is apparently not readily mobilized by these histamine liberators.

On the other hand, we found that some histamine liberators were moderately active in antagonizing the action of histamine on the guinea-pig's ileum. Of the compounds listed above, nos. 16 and 20 had some anti-histamine action in dilutions of 10^{-5} or less, nos. 14 and 23 were effective at the 10^{-4} level, and nos. 1 and 8 had no effect at this dilution. The anti-histamine effect of the compounds was not a very specific one; the action of acetylcholine on the gut was antagonized to about the same extent.

In the anaesthetized cat the injection of various histamine liberators, in doses sufficient to produce a large effect on the blood pressure, caused vigorous contractions of the intestines, visible through the abdominal wall. This is what would be expected in view of the large amount of histamine entering the blood, but reflex stimulation was not excluded.

Gastric secretion. This was measured in a cat anaesthetized with chloralose; the pylorus was tied and the vagi cut in the neck. Warm distilled water in 20 c.c. portions was introduced into the stomach through a catheter passed down the oesophagus, and was withdrawn after 10 min. for the titration of 'free' HCl (methyl red). The slow infusion of a licheniformin hydrochloride solution at a constant rate (0·24 mg./kg. min.) into a femoral vein resulted in an output of free acid, beginning between 10 and 20 min. after the start of the infusion, and outlasting it by about 20 min.; about 85 μM. of acid was secreted. The arterial pressure, which was simultaneously recorded, remained unchanged. The secretory effect would be expected from the ability of licheniformin to liberate histamine; a possible action through disturbance of the CO_2-bicarbonate balance (Browne & Vineberg, 1932) seems unlikely but has not been excluded. Feldberg & Holmes (1941) have made similar observations with 'curarine'.

Haematocrit. As Dale & Laidlaw (1918) showed, a large dose of histamine causes a rise in the haematocrit value. Later work (cf. Stead & Ebert, 1941; Gibson, Seligman, Peacock, Aub, Fine & Evans, 1945; Gibson, Seligman, Peacock, Fine, Aub & Evans, 1946) suggests that such effects as this may be due to the redistribution of blood between large and small vessels as well as to loss of plasma through the capillary wall. Whatever its mechanism, the effect of histamine liberators on the haematocrit value is the same as that of histamine. Fig. 11 shows the effect of a fairly large dose of diamino-octane dihydrochloride (8 mg./kg.) in a chloralosed cat.

Skin. Solutions of drugs for injection into human skin were made in isotonic (1·3%) $NaHCO_3$, so as to be approximately uniform in pH. The injections were made with a fine needle on the flexor surface of the forearm, the volume of fluid being about 0·02 c.c in each instance. The effect was rated in terms of an arbitrary scale, + + + representing a wheal of the maximum size with pseudopodial extensions, + + and + smaller wheals, ± a doubtful and − a negative effect. The size and intensity of the flare was not taken into account, but appeared to correspond with the degree of whealing. The three subjects gave very similar responses. The principal results are given in Table 4, and from these the following conclusions can be drawn.

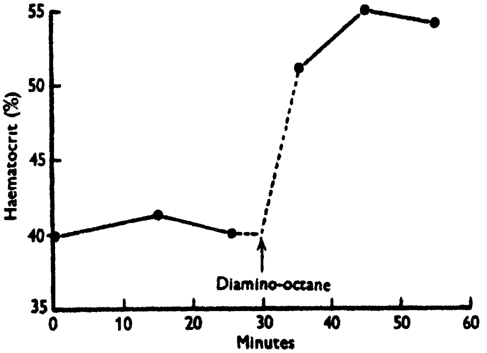


Fig. 11. Effect of the injection of 1:8-diamino-octane dihydrochloride (8 mg./kg.) on the haematocrit value in the cat.

TABLE 4. Whealing of human skin by histamine, histamine liberators and other compounds

No.	Compound	Conc.	Effect: Alone	Effect: With Neoantergan (5×10^{-4})	Effect: On previously injected site
1	Histamine	10^{-4}	+ + +, + + +	...	...
2	,,	10^{-5}	+ +, + +, + + +, + +	...	+ +, 6 hr. after histamine 10^{-4}; +, 24 hr. after licheniformin 10^{-3}
3	,,	5×10^{-6}	+ +, + +, +, + +	−, ±, ±	+, 24 hr. after licheniformin 10^{-4}; + +, 24 hr. after diamidinodecane 10^{-3}
4	,,	10^{-6}	+, +	...	...
5	,,	10^{-7}	+	...	...
6	,,	10^{-8}	−	...	...
7	Licheniformin	10^{-2}	+ + +, + + +	...	...
8	,,	10^{-3}	+ + +, + + +, + + +, + + +, + + +	... ...	... ...
9	,,	5×10^{-4}	+ +, + + +	±, +	±, 24 hr. after licheniformin 10^{-3}
10	,,	10^{-4}	+ +, + + +	...	+ +, 6 hr. after histamine 10^{-4}; −, 6 hr. after licheniformin 10^{-3}
11	,,	10^{-5}	+, +	...	−, 24 hr. after licheniformin 10^{-3}
12	,,	10^{-6}	+	...	...
13	Diamino-octane	10^{-2}	+ + +, + + +	...	...
14	,,	10^{-3}	+ +, + +, + +	...	...
15	,,	5×10^{-4}	+ +, +	±	±, 24 hr. after diamino-octane 10^{-3}
16	Diamidinodecane	10^{-3}	+ +, + + +, + +	...	...
17	Diguanidinopentane	10^{-2}	+ +, + +	...	...
18	,,	10^{-3}	+ +, +, + +	...	...
19	Diisothioureahexane	10^{-3}	+ +, + +	...	...
20	V 187	10^{-3}	+ +	...	...
21	Putrescine	10^{-3}	±	...	...
22	Benzamidine	10^{-3}	−, ±, ±	...	...
23	Neoantergan	10^{-3}	−, ±, −	...	...

Concentrations of histamine refer to the base; those of licheniformin, V 187 and benzamidine to the hydrochloride; those of Neoantergan to the maleate; those of the other compounds to the dihydrochloride.

(1) Compounds which were active histamine liberators, as judged from their effectiveness in the test on the cat's blood pressure, all produced a typical wheal-and-flare response identical with that evoked by histamine, when they were injected intradermally in high dilution (nos. 7–20). No persistent in-

flammation or necrosis was caused by any of the compounds in the concentrations used.

(2) Conversely, no wheal or flare was produced by related compounds (nos. 21, 22) showing no activity in the blood-pressure test.

(3) Neoantergan added to a histamine solution greatly reduced or abolished its effects on the skin (no. 3); it also counteracted the effects of histamine liberators (nos. 9, 15). Dews & Graham (1946) have shown that Neoantergan prevents the development of histamine wheals in human skin, and Parrot (1942) has found that the related base Antergan inhibits the wheal-and-flare response to stroking the skin, which is mediated, as Lewis (1927) demonstrated, by the liberation of histamine or a similar substance. It must be remembered, however, that local anaesthesia also reduces the wheal and abolishes the flare, produced by histamine (Lewis & Grant, 1924). Since Neoantergan is a powerful local anaesthetic (Dews & Graham, 1946), its inhibition of the cutaneous effects of histamine liberators may depend on this property rather than on its specific anti-histamine properties.

(4) An area of skin which has been the site of a wheal produced by a histamine liberator is refractory, for 24 hr. at least, to the production of a second wheal by the same agent (nos. 9, 10, 11, 15), although histamine is still effective (nos. 2, 3). Either histamine or a histamine liberator will produce a second wheal on the site of a histamine wheal (nos. 2, 10). A possible explanation, which we have not tried to verify, is that the local store of histamine remains depleted for a long time after the injection of a histamine liberator. A similar phenomenon was described by Grant, Pearson & Comeau (1935), who noted that skin areas in which whealing had been produced by warming or by a parasympathomimetic drug would not again form wheals in response to the same stimulus, unless an interval of about 2 days had elapsed.

It is clear that all these effects are most easily explained on the assumption that the compounds in question liberate histamine when introduced into the skin. If it be supposed that histamine, locally liberated in this way, has the same effect weight for weight as injected histamine, the amounts of histamine released can be roughly calculated. On this basis, for example, between 10 and 100 molecules of diamidinodecane are required to liberate a molecule of histamine.

Miles (1948) has observed that the intradermal injection of licheniformin, stilbamidine or V187 into a guinea-pig which had received pontamine blue intravenously is followed by a local blue discoloration of the skin, similar to that produced by an intradermal injection of histamine.

Blood pressure of the dog. Most of our experiments on the vascular effects of histamine liberators were done on cats. Dogs were used in a number of later experiments, the results of which differed somewhat from those observed in cats, and will therefore be described separately.

Each of the histamine liberators tested produced a sharp fall of blood pressure when given by vein to anaesthetized dogs, and in some animals the fall occurred after an interval of 20–25 sec., just as in the cat anaesthetized with chloralose. More often, however, it began within 10 sec. of the injection, and in that case it was sometimes possible to note a second phase of the depressor effect beginning some 15 sec. later. In one dog, the depressor response could be changed repeatedly from an immediate to a delayed one by the continuous infusion of adrenaline at a rate (6 μg./kg. min.) barely sufficient to elevate the blood pressure. The latency of the blood-pressure fall produced by diamino-octane was raised from 8 ± 1 sec. in the absence of adrenaline to 24 ± 3 sec. during the infusion, whereas the latency of the fall produced by histamine was constantly 5–6 sec. Individual dogs varied more than individual cats in their sensitivity to histamine liberators, but on the average were equally or slightly more sensitive: the nature and size of the depressor effect were not obviously related to the anaesthetic used. The enhanced response to the second of two closely succeeding injections of a histamine liberator, which has been described above for the cat, was not seen in the dog.

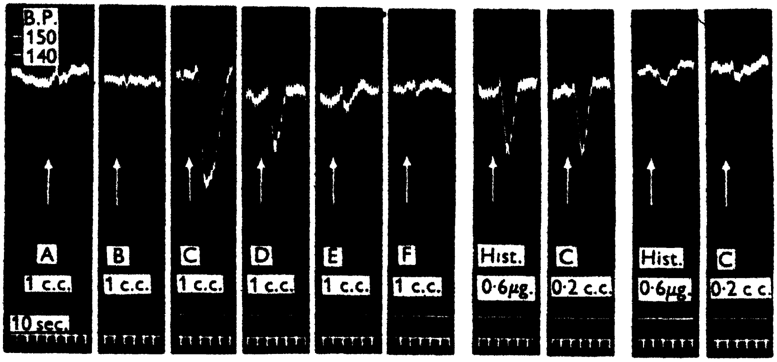


Fig. 12. Cat, chloralose: blood pressure. Effect of histamine and of heparinized plasma from a dog anaesthetized with chloralose-urethane. Hist., histamine. A to F, plasma samples; A and B, 20 and 10 min. before injection of diamino-octane (see text); C, D, E and F, 1, 13, 31 and 106 min. after the injection. Before the last two injections the cat received 0·3 mg./kg. of Neoantergan maleate.

Dog's plasma obtained after the injection of large doses of a histamine liberator contained histamine: the methods used for its identification and assay were those already described for histamine in cat's plasma. The observed increases in plasma histamine were even larger than those found in the cat. Thus the following values were observed in different dogs 1 min. after the injection of a liberator: 3·0 μg./c.c. after diamino-octane dihydrochloride (15 mg./kg.); and 2·0 μg./c.c. after diamidinodecane dihydrochloride (15 mg./kg.). Fig. 12

shows the depressor effects produced by successive plasma samples in the diamino-octane experiment.

When a histamine liberator was injected into a dog with opened abdomen, the liver could be seen to darken and to swell dramatically as the blood pressure fell, and to resume its normal appearance as the blood pressure recovered.

In a few dogs, for reasons which are unknown, the usual effects of histamine liberators on the blood pressure or on the plasma histamine level were either much reduced, or absent, or could be elicited only at the outset of the experiment.

Blood coagulation. We have never observed any retardation in the clotting of the blood of cats as the result of the administration of histamine liberators even in doses large enough to produce shock. In dogs, on the other hand, the clotting time of arterial blood has shown a striking increase in every experiment in which a release of histamine was observed. The data of one such experiment are shown in Fig. 13. It will be observed that the effect on clotting in this experiment did not become maximal until the plasma histamine had begun to fall, and this result was also obtained in the other experiments. Histamine liberators added to dog's blood *in vitro* had no effect on the clotting time. Histamine itself affects the clotting time very little, either *in vivo* or *in vitro*.

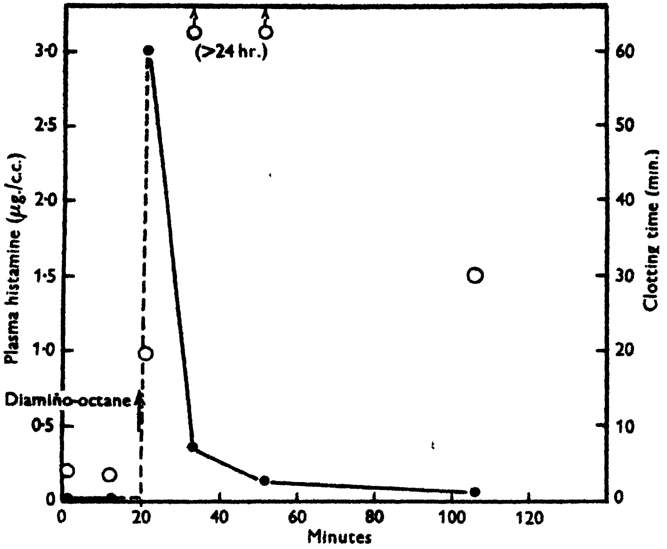


Fig. 13. Dog. Same experiment as Fig. 12. Effect of diamino-octane dihydrochloride (15 mg./kg.) on clotting time of arterial blood (circles) and on plasma histamine content (dots).

The effects of histamine liberators on the dog appeared to be similar to those observed in peptone and anaphylactic shock, in which the incoagulability of the blood is due to the presence of heparin (Wilander, 1938; Jaques & Waters, 1940). The clotting defect provoked by histamine-liberators is apparently caused in the same way. Toluidine blue is known to form with heparin a complex in which the dye assumes its metachromatic colour; the heparin so combined has no anticoagulant activity (Jorpes, Holmgren & Wilander, 1937). A sample of incoagulable blood from a dog which had received diamino-octane clotted within a few minutes after the addition of toluidine blue (0·075 mg./c.c. of blood), and plasma from such blood displaced the absorption spectrum of toluidine blue toward the violet in the same way as plasma to which heparin had been added. The evidence so far obtained thus suggests very strongly that histamine liberators in the dog are also heparin liberators; but final proof that this is so must await the isolation of heparin from the blood of dogs poisoned by such compounds.

The site of histamine release. We have emphasized that in the chloralosed cat there is no immediate effect of histamine liberators on the blood pressure, although there may be an abrupt and substantial fall after a characteristic latent period of 20–25 sec. This in turn implies either that the histamine is released into a small portion of the vascular bed, or that it enters the blood stream beyond the site of the main capillary resistance, or both of these. In some circumstances, however, the immediate depressor effect is more prominent; this is usually the case in the dog, and in the etherized or decerebrate cat, although these are not necessarily more sensitive to intravenous histamine than the cat anaesthetized with chloralose. An immediate vasodilator effect can also be demonstrated for the diamidines in the perfused limb of the cat (Wien, 1943), provided that the vascular tone is maintained with adrenaline. Possibly the presence or absence of an immediate depressor effect in the intact animal depends on the relative distribution of the peripheral resistance among the arterioles, meta-arterioles (Chambers & Zweifach, 1944) and capillaries. The modification by infused adrenaline of the depressor response in the dog, already referred to, can probably he explained in this way. As to the nature and location of the vessels into which the liberated histamine diffuses, our experiments give no suggestion.

More information is available as to the organs chiefly concerned in the liberation of histamine in the cat. We injected a small dose of licheniformin alternately into the left and right auricle of a cat whose chest had been opened under artificial respiration (Fig. 3). The depressor effect produced by injection into the left auricle was greater, and a few seconds earlier in onset, than that produced by injection into the right auricle. The *lungs*, therefore, do not contribute any large part of the histamine entering the blood (unless, as seems improbable, the histamine is liberated only in those parts of the lungs supplied by the bronchial arteries). The *liver* and *digestive tract* in the cat are also unimportant. Licheniformin injected into the coeliac or hepatic artery, or into the portal vein, had little effect on the blood pressure unless the dose was large. On the other hand, after evisceration a smaller dose of a liberator was needed to produce a depressor effect than before.

The injection of licheniformin into the central stump of the cut inferior mesenteric artery of an eviscerated cat, so that the drug reached mainly the skin and muscle of the hindlimbs, produced a fall of blood pressure larger than the same dose intravenously. This suggests that most of the histamine liberated in the cat comes from *skin* or *muscle*. We have little information about the relative importance of these two tissues. In the one experiment in which their histamine content was measured, that of the skin fell by 40 %, but there was no detectable change in that of the muscle. On the other hand, Wien (1943) observed that the intra-arterial injection of diamidines into a skinned limb was followed by a large fall of blood pressure, and we were able to produce a typical depressor effect in a cat which had been skinned as completely as possible.

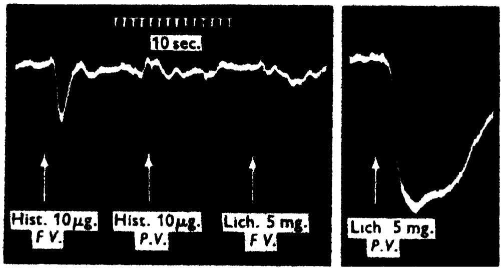


Fig. 14. Dog, sodium barbitone: blood pressure. Effect of injection of histamine (Hist.) and of licheniformin hydrochloride (Lich.) into the femoral vein (*F.V.*) and the portal vein (*P.V.*). The second dose of licheniformin was given 10 min. after the first.

Most of the experiments described above were repeated on the dog. Injection of a histamine liberator into the right auricle was no more effective than injection into the left auricle; and evisceration of the animal left the depressor response unchanged in character, though much reduced in size. These results correspond to those obtained in the cat, and suggest that in the dog the compounds under discussion can liberate histamine from skin and/or muscle, but not to any great extent from the lungs. We did, however, find one striking difference between the two species. In the cat, the liver does not appear to participate in the release of histamine, but in the dog it is the organ chiefly responsible. The evidence for histamine release by the dog's liver comes from two observations: (*a*) as has just been mentioned, the depressor effect of histamine liberators was much smaller after evisceration, although the depressor effect of histamine itself was not reduced; (*b*) injection of a histamine liberator into the portal vein of a dog produced a much greater effect on the blood pressure than injection of the same dose into a systemic vein, whereas histamine itself was less effective by the intraportal route (Fig. 14). These results were the opposite of those obtained in the cat, which became more sensitive to histamine liberators after evisceration and gave a bigger response to an ordinary intravenous injection than to an intraportal one.

Pharmacological similarity to peptone. In both the cat and the dog, the effects of histamine liberators closely imitate those of peptone, and, almost as closely, those of antigens in sensitized animals. Peptone, like histamine liberators but unlike histamine, lowers the dog's blood pressure more when given intraportally than when given intrajugularly (Feldberg, Schilf & Zernik, 1928), and is more effective in the intact than in the eviscerated dog (Feldberg *et al.* 1928; Mautner & Pick, 1929). The toxic effects of antigens in sensitized dogs are likewise well known to depend on the presence of the liver. Both these agents cause a reduction in the histamine content of the liver which apparently is sufficient to account for the concomitant increase in the histamine content of the body fluids (Ojers, Holmes & Dragstedt, 1941; Holmes, Ojers & Dragstedt, 1941). No doubt the compounds we have examined would similarly reduce the stored histamine of the dog's liver, but we have made no estimations of liver histamine.

In the cat, on the other hand, the liver plays no active role in the production of peptone shock (Feldberg, 1929) or of anaphylaxis (Edmunds, 1914); and we have found it equally unimportant in determining the action of histamine liberators.

It is well known that a dog which has recovered from peptone shock is little affected by a second injection of peptone. In the one experiment of this sort which we did, we found that the induced refractoriness to peptone also extended to the effects of a histamine liberator. A dog anaesthetized with chloralose-urethane received 400 mg./kg. of 'Difco proteose-peptone' from which the free histamine had been removed by treatment with Permutit. The dog went into shock; the plasma histamine level rose to 0·42 μg./c.c., and the blood became completely incoagulable. After 75 min., the plasma histamine had fallen below 0·03 μg./c.c. and the clotting power of the blood had returned to normal. A large dose of diamidinodecane dihydrochloride (10 mg./kg.) was then injected: it produced only a trivial rise of plasma histamine, to 0·05 μg./c.c., and no effect on the clotting time. Many previous workers have found that not only is a refractory state produced by an effective injection of peptone, but that a certain proportion of dogs are from the beginning insensitive to peptone. We have found this to be the case with histamine liberators as well.

A further similarity between the effects of peptone and those of histamine liberators has already been described: this is the incoagulability of the blood which both produce, and which we have tentatively ascribed to the liberation of heparin. We have not been able to produce this phenomenon in the cat.

Lastly, we have noted that in the dog histamine liberators, especially when injected into the portal vein, cause a rise in intraportal pressure and a great increase in the size of the liver. These effects, which depend on constriction of the hepatic veins, are produced by peptone, but also by histamine itself (Feldberg *et al.* 1928; Mautner & Pick, 1929) when given in large doses. Our impression is that the obstruction of the hepatic circulation produced by a histamine liberator is greater, in proportion to the fall of arterial pressure, than that produced by intravenous histamine, and so is to be ascribed, like the similar effect of peptone, mainly to the histamine set free within the liver itself; but we have no quantitative data on this point.

Substances modifying the action of histamine liberators

Anti-histamine drugs. It is somewhat difficult to predict how far these substances would suppress the vascular action of histamine liberators, even though this action is considered to be due solely to the histamine liberated. Neoantergan, Benadryl, and related compounds have only a limited ability to antagonize the depressor action of histamine in the cat and dog (Bovet & Walthert, 1944; Dews & Graham, 1946; Marsh & Davis, 1947). Moreover, the amount of histamine entering the blood when a dose of liberator is injected will generally be much greater than the amount of histamine required to produce an equal reduction in blood pressure when injected suddenly into a vein, as is shown by the greater duration of the effect in the former case. The extent to which histamine can lower the blood pressure depends not only on the plasma histamine concentration, but on the rate at which this is changing (cf. Emmelin, 1946). On the basis of such considerations it might be expected that the anti-histamine drugs would antagonize the circulatory effects of the histamine

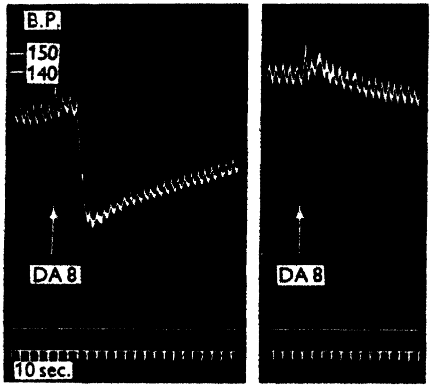


Fig. 15. Cat, chloralose, eviscerated: blood pressure. Effect of diamino-octane dihydrochloride (0·8 mg./kg.) before and after Neoantergan maleate (4 mg./kg.).

liberators less completely than those of histamine. Our experiments indicate that this is true to some extent, but we found, nevertheless, that Neoantergan abolished the depressor effect of a small dose of a liberator (Fig. 15), and retarded the rate at which the blood pressure fell when a larger dose of liberator was given. Further, when the blood pressure had been lowered by repeated injections of a liberator, it was partially restored by Neoantergan or Benadryl, which had no pressor action in the normal animal. Thus, anti-histamine drugs can antagonize the circulatory effects of histamine liberators, but the antagonism is a limited one.

Calcium salts. Wien (1943) observed that the injection of a solution of $CaCl_2$ (10–15 mg.) or calcium gluconate (50–100 mg.) reduced or abolished the depressor action of stilbamidine and propamidine in the cat. We tested the effect of these salts on the vasodilator action of licheniformin and propamidine: in the chloralosed cat, the inhibitory effect was usually small, and consisted mainly in slowing the rate at which the blood pressure fell after a dose of the histamine-liberator; but in the etherized cat calcium appeared to reduce the depressor action considerably.

Heparin. The effect of heparin on the vascular response to histamine liberators was examined because it was noted that some of the compounds form insoluble complexes with heparin, and because heparin is known to inhibit the release by peptone of histamine from rabbit blood cells (Dragstedt, Wells & Rocha e Silva, 1942), and probably also from dog liver (Rocha e Silva, Scroggie, Fidler & Jaques, 1947). In two cats we determined the effect of a large dose of heparin (40–60 mg./kg.) on the depressor response to histamine and to licheniformin: the former was unaffected; the latter was slowed and reduced in size.

Alkalis. In two experiments it was found that doses of licheniformin or diamidinodecane which produced a good depressor effect when dissolved in 1 c.c. of 0·9% NaCl, were nearly or quite inactive when dissolved in 1 c.c. of M/6 NaOH. The alkaline solutions recovered their activity on being neutralized. The phenomenon has not been studied further. The inhibitory effect of heparin and of calcium salts on the depressor response to histamine liberators is not a pH effect.

Splenin. Ungar (1945) has isolated from spleen a substance, apparently a polypeptide, which inhibits, in minute concentrations, the release of histamine in anaphylaxis and peptone shock. Through the courtesy of Dr Ungar we were able to test the effect of this substance, splenin, on the depressor action of licheniformin. Splenin was given intravenously in doses of 6–10 μg./kg. 5–20 min. before the injection of licheniformin, these doses being many times greater than that which Ungar found necessary to inhibit the release of histamine in guinea-pig anaphylaxis. The depressor response to the histamine liberator was unaltered.

Other substances. Raiman, Later & Necheles (1947) have reported that *rutin*, a flavone glucoside, injected intraperitoneally into sensitized guinea-pigs protects them against anaphylaxis; and its aglycone, *hesperidin*, is said to protect against anaphylactic shock (Hiramatsu, 1941). Each of these compounds, injected intravenously as the sodium salt in doses up to 50 mg./kg., failed to modify the depressor activity of histamine liberators in the cat.

Procaine hydrochloride (15 mg./kg.), putrescine dihydrochloride (12 mg./kg.), and a number of inactive monoamidines all failed to inhibit the response to subsequent injection of a histamine liberator.

The amount of histamine released

The question naturally arises whether the amount of histamine released when a histamine liberator is injected is sufficient to account for the effect of the latter on the blood vessels. We were unable with the methods available to detect any increase in plasma histamine associated with small depressor responses to the injection of either histamine or a histamine liberator. The data in the literature do not suggest any very close correlation between observed values for plasma histamine and the circulatory state of the animal: it is clear, indeed, that the vessels gradually become unresponsive to histamine while the plasma histamine remains at a high level, or even continues to rise (Emmelin, Kahlson & Wicksell, 1941; Emmelin, 1946). In two experiments on cats, we observed that a single intravenous injection of 1 mg./kg. of histamine, which caused a profound but not fatal lowering of the blood pressure, the plasma histamine level 10 min. after the injection was 0·5 μg./c.c.; and Code (1939) found in the dog that the histamine content of venous blood rose to 0·2–0·4 μg./c.c. 1 min. after an injection of 0·3–0·4 mg./kg. of histamine, which produced a similar effect on the blood pressure. It is of interest to note that the values obtained for histamine in dog's plasma during anaphylaxis and peptone shock (Code, 1939; Dragstedt, Mead & Eyer, 1938; Rocha e Silva & Texeira, 1946) are of the same order of magnitude, ranging from 0·2 to about 2 μg./c.c. according to the severity of the shock. In our experiments, the plasma histamine frequently reached very high levels—up to 0·7 μg./c.c. in the cat, and up to 3 μg./c.c. in the dog—when histamine liberators were injected in large doses. There seemed, therefore, good reason to believe that these animals, whose circulatory state was indistinguishable from that of animals poisoned by histamine, were suffering from histamine overdosage. In one or two experiments, however, in which we estimated the histamine in the plasma of cats which had received large doses of histamine, the blood pressure was not lowered so far nor for so long as was usual following an injection of a histamine liberator which caused an equal increase in plasma histamine. Such a discrepancy, if real, might have been due to differences in the distribution of histamine in the two cases, the same level of plasma histamine being associated with different levels of free tissue histamine in the two kinds of experiment. Alternatively, it is possible that histamine liberators do not merely liberate histamine but damage the blood vessels in some other way.

We have found, however, no evidence of any circulating depressor substance other than histamine itself. It seems clear, therefore, that a great part at least of the vascular effects of histamine liberators is in fact due to the histamine set free by them.

DISCUSSION

Our experiments show that many basic organic compounds are able, when present in low concentrations, to liberate histamine from mammalian tissues, and that some of the more striking pharmacological effects of such compounds can be ascribed to the histamine that they liberate. The chemical structure necessary and sufficient to confer histamine-releasing activity cannot as yet be stated precisely. So far the compounds found to be active can be divided into two classes: those in which two basic radicals are separated by a substantial inert moiety of predominantly hydrocarbon nature; and a series of benzamidine derivatives possessing a second polar group remote from the amidine radical.

Many compounds of these two classes have been synthesized and tested for chemotherapeutic activity (cf. King *et al.* 1938; Fuller, 1942; Andrewes, King, van den Ende & Walker, 1944). Of these, at least the trypanocidal diamidines (stilbamidine, propamidine, pentamidine) appear to liberate considerable quantities of histamine when they are given to man by vein in the usual dosage. Some of the transient side-actions of these drugs, including itching, colic, and fall of blood pressure, which have been noted by many observers (Adams, 1941; Saunders, 1941; Lawson, 1942; Lourie, 1942; Kirk & Henry, 1944), may be attributed with confidence to the histamine that is set free. It is desirable that potential therapeutic agents, whose chemical structure suggests that they might liberate histamine, should, before they receive clinical trial be tested on animals like the cat or dog whose blood vessels are sensitive to histamine. Such compounds may, of course, have other toxic effects, not obviously related to the release of histamine: thus, prolonged administration of diamidines causes in animals severe damage to the liver and kidneys (Broom, 1936; Devine, 1940; Wien, Freeman & Scotcher, 1943); the same is true for diguanidines (Blatherwick, Sahyun & Hill, 1927; Bischoff, Sahyun & Long, 1929). Whether this sort of toxicity is dependent on the same types of chemical structure responsible for histamine-releasing activity has yet to be determined.

Our attempts to discover methods of counteracting the effects that we have ascribed to histamine liberation have not been very successful. The antihistamine substances now available are remarkably active in preventing many effects of injected histamine, especially those on smooth muscle; but they seem, for reasons we have discussed above, to be less efficient in preventing the vascular effects of histamine liberators. Of the substances which appear to modify the release of histamine, rather than its action, heparin possibly acts by forming complexes of low solubility with the liberators, but the action of calcium salts and alkalis cannot be explained in this way. Heparin is known to have some inhibitory effect on anaphylaxis and on the similar phenomena produced by peptone, both in the whole animal and in isolated tissues (Kyes & Strauser, 1926; Williams & van de Carr, 1927; Dragstedt *et al.* 1942), and calcium has been reported to act in a similar manner (Schittenhelm, Erhardt & Warnat, 1928; Kallós & Kallós-Deffner, 1935).

The release of histamine in our experiments is most simply explained on the supposition that histamine can be displaced by chemically similar bases from its combination with tissue substances. The histamine-liberating effects of diamines and diamidines would be easy to understand on this view, since histamine itself may be regarded as a diamine or an amidine in structure; and the biochemical resemblance of such compounds to histamine is illustrated by the fact that diamine oxidase destroys histamine (Zeller, 1938*a*) and is strongly inhibited by diamidines (Blaschko & Duthie, 1944) and diguanidines (Zeller, 1938*b*). This displacement hypothesis, however, cannot readily explain the ability to release heparin in the dog, which seems to be a characteristic property of histamine liberators: for heparin is not a base, nor is it liberated by histamine. It could perhaps be supposed that histamine and heparin are associated in some tissue complex, and that the displacement of the former mobilizes the latter as well, but we know of no evidence for such an association.

In the dog, as is well known, anaphylactic and peptone shock are also accompanied by the liberation of both histamine and heparin. It is attractive to suppose that a similar mechanism is responsible for the shock produced by histamine liberators. We have made two observations which support this supposition. Thus in the dog (but not in the cat) the liver is the 'shock organ' and the main source from which histamine is released in peptone shock and anaphylaxis, and the same is true for shock produced by a histamine liberator. Moreover, a dog which has just recovered from peptone shock cannot be shocked again by treatment with either peptone or a histamine liberator. It may be significant that the licheniformins, which are among the most active histamine liberators we have examined, are polypeptides containing a high proportion of basic amino-acids; the pharmacologically active agents in peptone may have a similar constitution.

Rocha e Silva and his co-workers in a series of recent papers (Rocha e Silva, 1941, 1944; Dragstedt *et al.* 1942) have revived in a modified form the theory that the symptoms of anaphylaxis depend on the activation, consequent on the union of antigen and antibody, of some proteolytic enzyme, which then splits off histamine or other toxic substances from the tissue proteins to which they are bound. Such an activation has been demonstrated for the fibrinolytic enzyme of dog's plasma in anaphylactic and peptone shock (Rocha e Silva & Texeira, 1946; Rocha e Silva, Andrade & Texeira, 1946; Scroggie, Jaques & Rocha e Silva, 1947); and the striking resemblance of the shock produced by trypsin injections (Rocha e Silva, 1944) to peptone and anaphylactic shock suggests that a generalized activation of certain proteases might acount for many of the changes observed in the two last-named conditions. We have not as yet studied the activity of plasma fibrinolysin or other proteases in dogs treated with histamine liberators, but the pharmacological similarity between these compounds and peptone suggests that such a study might be worth while. Besides the displacement hypothesis, therefore, the possibility must be considered that the histamine liberators act by unmasking tissue or plasma proteases. Our findings are so far indecisive on this point, which it should be possible to settle experimentally. We wish rather to draw attention to the fact, which we think of considerable significance, that there exists a large group of organic bases whose action reproduces the salient features of anaphylaxis and peptone shock.

SUMMARY

1. Many organic bases have the property, when given by vein to the cat, of producing a sudden fall of arterial pressure, the beginning of which is delayed for some 20–25 sec. after the injection.

2. Blood or plasma obtained during the period of lowered blood pressure contains a depressor substance whose action is manifested within a few seconds after injection. This substance has been identified pharmacologically as histamine, and it is released in amounts sufficient to account for the vascular effects of the bases which liberate it.

3. Among the bases found to liberate histamine were diamines, diamidines, diguanidines, diisothioureas, diquaternaries, some benzamidine derivatives, and licheniformin, an antibiotic polypeptide.

4. Such compounds elicit a typical triple response when injected into human skin. When continuously infused by vein into a cat they evoke a secretion of gastric juice.

5. In the dog these compounds likewise lower the blood pressure by releasing histamine in large amounts, and in addition they decrease or abolish the coagulability of the blood. The latter effect appears to be due to the liberation of heparin.

6. The liver is the main site of histamine release in the dog. In the cat, the main sites of the release are skin and muscle, and the liver is not involved.

7. The vascular effects of histamine liberators are reduced but usually not prevented by anti-histamine drugs. A variety of substances, including heparin, calcium salts and alkalis, also reduce these effects, apparently by interfering with the histamine release.

8. Possible mechanisms for the release of histamine by such compounds are considered, with particular reference to the similarity of their effects to the phenomena of anaphylaxis and peptone shock.

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THE EFFECT OF PRESSURE ON NERVE CONDUCTION AND NERVE-FIBRE SIZE

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The purpose of this investigation was to determine whether the effect of circumferential pressure on the size of a nerve could be correlated with its effect on conduction, and in what way these effects were altered by ligature of the vessels supplying the nerve.

Bentley & Schlapp (1943) found that conduction was blocked in the sciatic nerve of the cat by pressures of 130 mm. Hg and over in 3 hr. In their experiments the nerve was compressed between rubber membranes inflated with air. After the application of pressures which completely blocked conduction, they report complete absence of recovery on removal of the pressure, and they conclude that such pressures applied directly to the nerve probably produce a block of conduction by 'deformation of nerve substance at the boundaries of the compressed part' (p. 81). Denny-Brown & Brenner (1944*b*), using metal clips to compress the sciatic nerves of rabbits, found that with small pressures (2–7 g. clips) conduction was unimpaired even after several days, but higher pressures (170–430 g. clips) produced paralysis, lasting 5–18 days, when the clip was left in place for 2 hr. They considered the effect on the nerve to be due entirely to ischaemia. In a previous paper they state 'the compartmented physical structure of a large nerve, like the sciatic, is such as to protect some of the longitudinal vessels from external pressure' (Denny-Brown & Brenner, 1944*a*).

Edwards & Cattell (1928) state that 'the influence of pressure in blocking conduction is entirely dependent upon deformation of the tissue due to its unbalanced action'. Weiss & Hiscoe (1948), in describing acute experiments on the nerves of the rat compressed by arterial sleeves, state: 'The first marked effects appear only after several hours. They consist of a narrowing of the fibres in the constricted stretch'.

Gasser & Erlanger (1929) blocked the *A* fibres in the sciatic nerve of the frog with pressure of 25 lb./sq.in. The pressure was applied after threading the nerve through a vascular sleeve in the manner used by Meek & Leaper (1911). In a paper dealing with the effects of asphyxia produced by surrounding a nerve with hydrogen, Cooper (1923) suggested that the oxygen was not essential for the transmission of the impulse, but was required for the oxidation of breakdown products. In a later paper Gerard (1930) did not support this view, and suggests, from the results of his many observations on the effect of an atmosphere of nitrogen on conduction, that 'normal membrane potentials (and other properties) are dependent on oxidations'.

Investigating the sensory changes in the human arm Lewis, Pickering & Rothschild (1931) found that, with their special clamp which compressed the nerves locally but did not occlude the circulation to the limb, they could produce sensory changes with pressures of 60–70 mm. Hg, but that sensory changes were not produced when an encircling cuff was placed round the limb and inflated to a similar pressure. Localized pressure equal to 150 mm. Hg on the ulnar nerve caused a muscular palsy within 30–40 min. which they attributed to a loss of blood supply within the compressed region of the nerve and not to direct pressure on the nerve fibres.

In a previous paper (Causey, 1948) it was shown that circumferential pressure could be applied to a nerve by direct mercury pressure in a glass chamber sealed with a gel of sodium alginate, and that pressures of 80 mm. Hg and over produced a decrease of 28% in the cross-sectional area of the nerve at the site of pressure; part of this decrease was due to a decrease in the mean fibre size, and a larger part to compression of the interstitial area. It seemed that further evidence on some of the points quoted above could be obtained by using the mercury chamber for the application of pressure, and determining the acute effect of localized pressure on nerve size, nerve-fibre size, and conduction.

METHOD

All the experiments were carried out on the nerve to the medial head of the gastrocnemius (N.G.M.) of rabbits, anaesthetized with nembutal and ether. All the rabbits were healthy adults; no attempt was made at uniformity of sex, weight or breed. The general method used will be described under two heads: (1) a method of applying circumferential pressure to the nerves, (2) a method of preparation of photographs of sections for measurement and counting.

(1) A small glass chamber (2·5 × 1 cm.), with an open top, was made at the end of a glass tube; the tube was connected to a mercury manometer by rubber tubing with a control clamp between the chamber and the manometer; the whole was filled with mercury. In the top of the sides of the glass chamber were two smooth grooves 1 mm. in depth. The N.G.M. was dissected from the main tibial trunk and the chamber slipped underneath it from the side, so that the nerve lay at the bottom of the grooves. The top of the chamber and the grooves were coated with a gel made of sodium alginate and water. A closely fitting lid was coated on the edges of its undersurface with the same gel and clamped firmly in place on top of the chamber. When the nerve was placed well down in the grooves, release of the control clamp caused the mercury to pass between the nerve and the lid and circumferential compression of a short segment of the nerve was obtained. The height of the mercury head above the level of the nerve was varied from 45 to 670 mm. in different experiments. The stimulating electrodes, consisting of two silver wires embedded in perspex, were placed under the nerve, proximal to the chamber. Muscle response was either observed visually or recorded on smoked paper by a pointer attached to a torsion wire. The tendon of the medial head of gastrocnemius was separated from the calcaneal tendon and attached to the recording lever by a hook and thread.

(2) By placing padding underneath the operated limb a trough was formed between the femur and the biceps muscle, in which the nerve ran longitudinally. At the end of the observational period a modified Flemming's solution, consisting of 15 c.c. 1 % chromic acid, 4 c.c. 2 % osmic acid and 1 drop of glacial acetic acid, was poured into the trough around the nerve. After maintaining the pressure on the nerve for 5 min. the control clamp was closed and the chamber removed from the nerve. Fixation was continued *in situ* for a further 25 min. The N.G.M. was then removed and fixation continued in a tube of Flemming's solution, with a waxed cork, for 24 hr.

After dehydration in alcohols the nerve was embedded in paraffin, cut at 5 μ. and stained by a modified Weigert technique (Gutmann & Sanders, 1943). Sections were selected from sites above, at, and below, the region of pressure and photographed direct on to bromide paper at × 750 magnification. On these photographs measurements were made of the total cross-sectional area of the nerve bundle by means of a planimeter, the mean of three readings being used. The nerve fibres were measured by fitting each fibre into a series of circles marked on a thin sheet of perspex; each circle represented the area corresponding to diameters of 2, 4, 6, 8, etc., μ multiplied by 750. Each fibre was pricked off with the needle of a mechanical counter as it was fitted into its size group. A fibre count was thus obtained classifying the fibres into groups at 2 μ. intervals. The root mean square diameter (D) was used to give a weighted mean (Sanders & Young, 1946). Only myelinated fibres were measured and the total diameter of the fibre (axon and myelin) was used. In control experiments it was found that the difference in D at different levels in the same nerve showed a mean of 0·17 μ. with a standard deviation of $\pm 0{\cdot}13$ μ.

Block of conduction and nerve-fibre diameter. In a series of fourteen rabbits, pressures varying from 45 to 670 mm. Hg were applied for 10 min. (in some of the cases submitted to higher pressure the time was varied). The nerve was stimulated proximal to the pressure chamber before and at the end of the pressure period, by short bursts from an induction coil, the strength of the stimulus being adjusted, before the application of the pressure, to produce a maximal twitch. The presence or absence of a muscle twitch was noted and fixative solution then poured round the nerve as described above. The N.G.M. of the opposite limb was dissected out and fixed in the same manner, without the application of pressure. The root mean square diameter above, at, and below, the region of pressure and the total nerve-bundle area were determined for the compressed nerve. The root mean square diameter and nerve-bundle area of the control nerve were also determined to show that there was a diminution in size at the site of pressure when compared with the control nerve as well as in relation to the other levels in the same nerve.

RESULTS

Pressures of 125 mm. Hg and over produce a significant decrease in the mean fibre diameter at the site of pressure, when this diameter is compared with the mean diameter either above or below the site of pressure, or when compared with the mean diameter of the control nerve. In all these cases there was no visible muscle twitch at the end of the period of pressure (Table 1). When the muscle twitch is recorded on a kymograph (see Table 2) the time needed for complete obliteration of the recorded twitch is found to be longer, with an average of 18 min.

In Table 1 the results for all fourteen animals are tabulated. Below 100 mm. Hg pressure there is no blockage of conduction, but there is a small decrease in fibre size, even at 75 mm. pressure. Between 100 and 125 mm. Hg pressure there is variation, both in the effect on fibre size and in the effect of conduction in different animals. The mean of the differences between the root mean square diameters of the fibres at and below the region compressed for those nerves in which conduction was blocked is 1·4 μ., or 12% of the mean of the root mean square diameters of the control nerves. The total area of the nerve bundle, measured with a planimeter, showed for all nerves in which conduction was blocked, a mean decrease in size of the nerve bundle in the compressed region as compared with the area of the nerve bundle below the compressed region. This decrease amounted to 26% of the mean area of the control nerves.

TABLE 1. Root mean square diameter (*D*) measured above, at, and below, the region of pressure, and from a control nerve of the opposite limb. + indicates muscle response on stimulation of the nerve proximal to the pressure.

Rabbit no.	Pressure (mm. Hg)	Time (min.)	*D* above press	*D* at press	*D* below press	*D* control	Conduction
1	45	10	13·2	13·2	13·5	13·2	+
2	50	10	11·2	11·7	11·1	12·2	+
3	75	10	13·0	12·2	12·7	13·0	+
4	100	10	11·7	10·7	11·8	11·7	−
5	100	10	11·7	11·6	11·9	11·8	−
6	105	10	14·2	14·1	14·1	14·1	+
7	125	10	11·7	10·2	11·5	11·1	−
8	150	10	10·8	9·8	11·2	10·5	−
9	250	10	13·1	11·3	13·4	11·4	−
10	370	30	12·5	11·6	13·0	11·9	−
11	470	7	12·0	11·3	12·2	12·3	−
12	500	8	12·4	11·3	12·6	12·1	−
13	560	15	12·0	10·5	12·0	11·6	−
14	670	10	13·7	11·7	14·4	12·7	−

The time required to produce complete block

To investigate more accurately the time necessary to block conduction a further series of experiments was carried out in which the muscle response was recorded throughout the experiment. The method of application of pressure and the histological technique were identical with the first series, but when the N.G.M. was dissected out, the tendon of the medial head of the gastrocnemius was separated from the calcaneal tendon and the muscle belly of the medial head separated, almost to the knee, from the lateral head. The tendon was then attached by a hook and thread to a lever mounted on a torsion wire. The excursions of the lever were recorded on a slow-moving drum. The stimulating electrodes were held in a clamp and left in place throughout the experiment. The stimuli were square waves of 1 msec. duration, at approximately one every 4 sec., the voltage was adjusted at the beginning of each experiment to give a maximal twitch. The opening of the control clamp between the manometer and the pressure chamber was marked on a base-line by a manually operated switch. Release of pressure was marked on the same base-line.

With pressures of 100 and 150 mm. Hg no failure of muscle response was recorded in 49 and 54 min. respectively. Fig. 1*a* shows the record at the beginning of compression by 100 mm. Hg and 48 min. later in the same preparation. There is only a slight decrease in the height of the twitch recorded. But in another animal (no. 16) block was obtained with 150 mm. Hg pressure in 20 min. With all pressures from 200 to 400 mm. Hg there was failure of conduction. The average time required for the development of block in fifteen experiments was 20 min. All the nerves recovered their ability to conduct an impulse when the pressure was released by removal of the lid of the chamber (see below). The mean time for recovery was 17 sec.

In Table 2 the times of onset of block and of recovery in twenty experiments are shown. The times required to produce block vary from 35·5 min. for a pressure of 300 mm. Hg (rabbit no. 25) to 3·6 min. for a pressure of 400 mm. Hg (rabbit no. 27). In this latter case the nerve had been blocked by the same pressure in 16·5 min. 2 hr. before, and allowed to recover (Barlow & Pochin, 1948). The onset of block was affected by the length of exposure and the amount of manipulation; thus, in cases where the chamber had to be reapplied because of leakages at the seals there was a tendency for a shorter time interval before

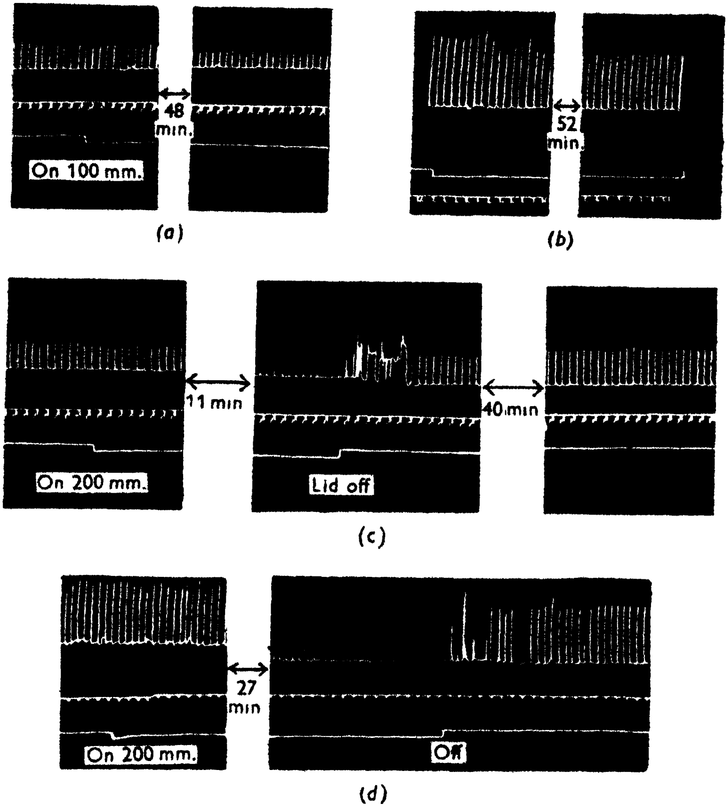

Fig. 1. Kymograph records of twitch from medial head of gastrocnemius of rabbit. Square wave stimulus of 1 msec. duration at intervals of 4 sec. Time marker 5 sec. The figure between the portions of tracings shows the approximate time interval of the deleted portion of the record. (*a*) At time of application of 100 mm. Hg pressure and 48 min. later. (*b*) At the beginning and end of local application of air pressure to the N.G.M. under a head of 240 mm. Hg. (*c*) At time of application of 200 mm. Hg pressure, followed by failure of muscle response after 11 min., recovery of muscle response on removal of lid of chamber and maintenance of response 40 min. later. (*d*) After application of 200 mm. Hg pressure. Failure of twitch after 27 min. and recovery on removal of lid of chamber.

block than in the cases where the first application of the chamber was successful. Reapplication of pressure following a previous block and recovery reduced the time preceding onset of block.

TABLE 2. Duration of pressure required to produce block, and time for recovery of conduction

Rabbit no.	Pressure (mm. Hg)	Time for onset of block (min.)	Time for recovery (sec.)	Rabbit no.	Pressure (mm. Hg)	Time for onset of block (min.)	Time for recovery (sec.)
15 L	100	Not blocked in 49 min.		21 L	270	13	20
16 L	120	Not blocked in 50 min.		22 L	270	24	5
15 R	150	Not blocked in 54 min.		23 R	280	18	22
16 R	150	20	10	24 L	290	14	5
17 R	200	24	5	24 R	300	18	22
18 L	200	6	10	25 L	300	36	5
19 L	240	29	40	25 R	300	21	5
19 R	250	21	40	26 L	400	9	20
20 R	250	27	65	27 R	400	17	5
21 R	270	16	5	27 R (2 hr. later)	400	4	53

Fig. 1*c*, *d* show sections of the continuous records taken from preparations with the nerves subjected to 200 mm. Hg pressure. The height of the record slowly diminishes after opening the control clamp and after 12 and 24 min. there is no further twitch. On removal of the lid of the chamber and release of pressure there is a very rapid recovery of the muscle twitch. In Fig. 1*c* a further record is shown of the muscle twitch 40 min. after the release of pressure, at which time the height of the record is fully maintained.

Experiments were also carried out in which the nerve was surrounded by air under pressure produced by a column of mercury. The glass chamber and distal portion of the manometer tubing were left full of air when the N.G.M. was placed in position in the groove and the lid clamped in position, on opening the control clamp the manometer level first of all fell rapidly as the air was compressed, but equilibrium was quickly attained and by suitable adjustment of the amount of air left in the tubing it was possible to have the nerve completely surrounded by air under pressure. This equilibrium was difficult to maintain, but it was possible to show that there was no block of conduction within the time interval which would cause block with mercury surrounding the nerve. Fig. 1*b* shows the tracing taken at the beginning of air pressure and 52 min. later, when the mercury head was 240 mm. The height of the twitch has only diminished by 5% and there is no evidence of block at a time well beyond the maximum time for complete block with mercury pressure.

Recovery of nerve-fibre size

The rapid recovery of conduction, after release of pressure by removing the lid of the chamber, has already been mentioned. In view of this the nerve size and nerve-fibre size after recovery were investigated. In three rabbits the left N.G.M. was dissected out and submitted to pressures of 100, 210 and 440 mm. Hg respectively. After 10 min. compression the trough in the limb was flooded with

Flemming's solution, whilst the pressure was maintained for a further 5 min. On the right side of the same animals the same pressures were applied for the same time, the chamber was then removed, the nerve was left for 10 min. and then flooded with Flemming's solution.

The root mean square diameters above, at, and below, the site of pressure, in these six nerves are shown in Table 3, and the total nerve areas at the same levels in Table 4. These figures show that with a pressure of 100 mm. Hg there is no significant difference either in the nerve size or nerve-fibre size between the three levels on the two sides. With pressures of 210 and 440 mm. Hg there was a decrease both in the nerve size and in the nerve-fibre size in the nerves when fixed with the pressure maintained, but the nerves released before fixation showed the same nerve size and nerve-fibre size at all levels.

TABLE 3. The root mean square diameter (D) when pressure was maintained during first 5 min. of fixation and when pressure was released before fixation

			Pressure maintained			Pressure released		
Rabbit no.	Pressure (mm. Hg)	Time (min.)	D above press	D at press	D below press	D above press	D at press	D below press
5	100	10	11·7	11·6	11·9	11·9	11·8	11·8
28	210	10	13·6	12·2	13·4	13·3	13·3	13·6
29	440	10	11·2	10·0	11·1	10·8	10·9	10·8

TABLE 4. The total nerve area at × 750 magnification when pressure was maintained during first 5 min. of fixation and when pressure was released before fixation

			Pressure maintained			Pressure released		
Rabbit no.	Pressure (mm. Hg)	Time (min.)	Area above press (cm.^{2})	Area at press (cm.^{2})	Area below press (cm.^{2})	Area above press (cm.^{2})	Area at press (cm.^{2})	Area below press (cm.^{2})
5	100	10	460	464	462	529	521	475
28	210	10	484	408	482	535	502	528
29	440	10	451	313	444	413	392	423

Recovery of conduction

The recovery of conduction after removal of the pressure was investigated: (1) On release of pressure and admission of air around the nerve by closing the control clamp and removing the lid of the chamber. (2) On release of pressure by dropping the manometer, so that the head of mercury was at the level of the chamber, and thus with the control clamp open the pressure on the nerve was reduced to zero. The nerve was still surrounded by mercury and by the alginate seals for a distance of 1 cm. (3) The manometer was lowered and the mercury was emptied out of the whole system so that pressure was removed and air admitted to all parts of the nerve except the small areas covered by alginate seals. Continuous records were taken as before.

Release of pressure by removal of the lid involved first closure of the clamp between the manometer and the chamber, then release of the clamp holding the lid of the chamber in place, and finally the removal of the lid. The moment of removal of the lid was recorded as the time of release of pressure. All the nerves recovered their ability to conduct an impulse as soon as the lid of the chamber was removed. The mean recovery time was 17 sec. Table 2, column 4, shows the estimated time of recovery in seventeen preparations. The times vary from less than 5 to 65 sec. This recovery time is so short that it is clear that, with the record on a slowly moving drum and the lack of a clear-cut end-point in a manipulation which takes a few seconds, they must be approximate. The average recovery time recorded of 17 sec. is therefore only an indication of the order of the recovery time.

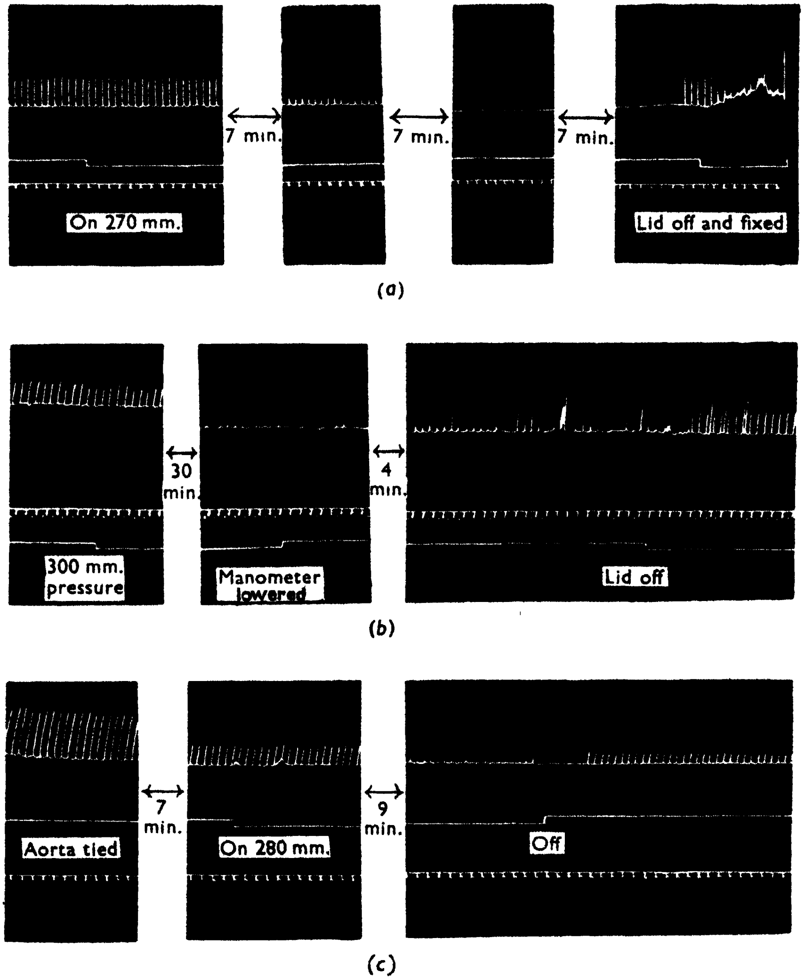

Fig. 2. (*a*) Effect of application of 270 mm. Hg pressure. No recovery 7 min. after dropping manometer pressure to zero. Recovery on removal of lid of chamber. Fixative solution then poured round the nerve. (*b*) Failure of muscle twitch 30 min. after application of 300 mm. Hg pressure. Partial recovery 4 min. after lowering of pressure to zero. Complete recovery after removal of lid. (*c*) Aorta and pulmonary artery tied at the base of the heart. 280 mm. Hg pressure applied locally, 7 min. later. Failure of muscle response after 9 min. and recovery on removal of lid.

When the manometer was dropped and the pressure reduced to zero recovery was delayed and showed marked irregularity. Fig. 2*a* shows records from a preparation in which, after obtaining block in 16 min., the manometer was dropped. The muscle gave no response for 10 min., at the end of which period removal of the lid was followed by immediate recovery. In Fig. 2*b*, however, after obtaining block in 36 min. the manometer was lowered to zero pressure, and after a period of 6 min. there was an incomplete recovery of the muscle twitch. On removal of the lid the twitch became equal to that obtained at the beginning of the record.

When the mercury was emptied out of the manometer and chamber, the recovery of the twitch was complete. The time and manipulation involved in removing the mercury in this way made it difficult to estimate the time of recovery with any accuracy.

Blood supply

The vascular supply of the sciatic nerve of the rabbit has been fully described by Adams (1942). Large, longitudinal vessels come from the inferior gluteal and popliteal arteries and are reinforced by small lateral vessels from the perforating and muscular branches. In the present experiments the latter small branches were divided in dissecting the N.G.M. from the tibial nerve, but the longitudinal vessels remained. Ligature of the gluteal and popliteal arteries, ligature of the common iliac artery, or ligature of the abdominal aorta, with or without the inferior vena cava, did not appreciably alter the time at which block occurred under mercury pressure and even after these ligatures conduction recovered within seconds on removal of the lid of the chamber. To exclude any possibility of a blood supply by way of the vessels of the abdominal wall or spinal cord the aorta was ligated as it leaves the left ventricle. The muscle and nerve were dissected out first, then the chest was opened in the middle line and a double thread passed through the transverse sinus of the pericardium on an aneurysm needle, and tied firmly round the aorta and pulmonary artery. This method of stopping the circulation seemed most suitable for these experiments because (1) the time of blockage of the circulation could be recorded with accuracy; (2) it avoided the possible poisoning of the nerve by overdose of chloroform or other narcotic; and (3) it avoided local exsanguination.

In trial experiments without localized pressure it was found that muscle twitches of diminishing height were obtained for 30–40 min. after tying the ligature round the aorta. When localized pressure was applied to the N.G.M. after tying these vessels it was found that the twitch was rapidly obliterated, but recovered, though diminished in height, on release of the pressure. The time taken by these phenomena varied considerably, depending on the interval between the placing of the ligature and the successful application of pressure. Table 5 shows the time relation of the events in a typical experiment in which a pressure of 250 mm. Hg was applied 5 min. after the ligature of the aorta. Conduction was blocked in 10 min. and recovery occurred 7 min. after the release of pressure.

In rabbit no. 30, with a pressure of 250 mm. Hg, block was obtained in 10·5 min. and reappearance of twitch within 20 sec. of release of pressure, whilst in rabbit no. 31 a pressure of 220 mm. Hg blocked conduction in

4·5 min. and 4 min. elapsed before the return of a recorded twitch. Fig. 2*c* shows sections from the record of rabbit no. 30. A steady decrease of the height of the twitch is seen between the time of tying the aorta and the beginning of the application of pressure. This decrease in height continues for 10 min. until there is no recorded twitch. Within a few seconds of removal of the lid of the chamber the muscle twitch returns, even though there is no circulation.

TABLE 5. Time-table of the course of events when local pressure was applied to the N.G.M. after ligature of the aorta and pulmonary arteries at the base of the heart

Rabbit no. 32	Nerve to the medial head of gastrocnemius dissected out and threshold determined
12.17 p.m.	Aorta and pulmonary artery tied
12.20 p.m.	Nerve conducting throughout its length
12.22 p.m.	Pressure of 250 mm. Hg applied
12.32 p.m.	No muscle reaction on stimulation *proximal* to chamber Muscle reaction on stimulation *peripheral* to chamber
12.33 p.m.	Pressure chamber removed
12.40 p.m.	Nerve conducting throughout its length

DISCUSSION

On application of localized circumferential pressure to a small nerve bundle in the rabbit, decrease in the total nerve size and in fibre size is first demonstrable at pressures between 80 and 100 mm. Hg. Block of conduction can be demonstrated, in acute experiments, with pressures of 150 mm. Hg and over, and recovery from such block occurs rapidly in all cases when the pressure is removed and air is admitted to the nerve. These observations clarify certain discrepancies in the findings of previous investigators which are quoted in the introduction to this paper. If a nerve, with a circular or ovoid cross-section, is compressed between two rigid surfaces, its axis parallel to the compressing surfaces will become extended, and the applied pressure will not be evenly distributed throughout the nerve bundle. With the fluid pressure used in the present experiments the transverse section of the compressed region remains circular (Causey, 1948) and the pressure is uniformly distributed around the periphery of the bundle. The pressure gradient is along the radius of the section, causing closer packing at the periphery than at the centre of the nerve. By using the N.G.M. the compartmented nature of the sciatic nerve has been avoided and the protection of longitudinal vessels afforded by this type of structure (Denny-Brown & Brenner, 1944*b*) cannot occur. These factors probably explain the much longer time interval observed by these authors and also by Bentley & Schlapp (1943), before the onset of block, and the absence of rapid recovery in their experiments.

Narrowing of the fibres in the constricted stretch of nerve was found by Weiss & Hiscoe (1948), only after several hours. Our experiments indicate the onset of narrowing very much earlier and are not in accordance with their suggestion that the endoneurial fluid is in a state of very high hydrostatic pressure. The gradual diminution of the muscle twitch before complete block, shown in the tracings, is presumably due to the peripheral fibres being affected before those in the centre of the nerve.

The reduction in nerve-bundle size and nerve-fibre size which results from the application of pressures sufficient to produce block of conduction, and the recovery after release of pressure show that there is deformation of nerve substance, as suggested by Bentley & Schlapp (1943). But the observations on the greater relative rate of recovery when the compressed region is exposed to air than when the pressure is released without admitting air to the compressed region, indicate that it is the presence of oxygen, and not the recovery of shape, which is essential to the recovery of conduction. This is supported by the observation that pressure on the nerve by air does not produce block within 52 min.

The results of the experiments reported here find their closest parallel in the results obtained by Lewis *et al.* (1931), in experiments on motor and sensory changes in the human arm. With localized pressure of 150 mm. Hg on the ulnar nerve they observed muscular paralysis in 30 min., with recovery in 20–30 sec. Our results suggest that, at least in experimental animals, the primary condition is one of local anoxia caused by local deformation of the nerve bundle, and recovery is rapid when the nerve again obtains access to oxygen.

SUMMARY

1. Local application of pressure by mercury to a nerve caused failure to conduct an impulse to the muscle when the stimulus was applied above the site of pressure. All pressures above 150 mm. Hg produced block. The average time required to obliterate the muscle response was 20 min.

2. Recovery of conduction took place within 5–65 sec. after the release of pressure and the admission of air.

3. Nerve-fibre size and nerve-bundle size were diminished at the site of pressure, and there was recovery of nerve-fibre size and nerve-bundle size 10 min. after the release of pressure.

4. Application of pressure by air did not cause failure to conduct an impulse in 52 min.

5. The circulation of the blood was stopped by ligature of the aorta and pulmonary artery at the base of the heart. After this was done it was still possible to block conduction along the nerve by localized pressure, and to obtain recovery on the release of pressure, before the final death of the preparation.

6. It is concluded that, in acute experiments, the failure to conduct an impulse after local pressure, is caused by local anoxia.

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THE EFFECTS OF INTRA-ARTERIAL ADRENALINE ON CARBOHYDRATE METABOLISM IN MAN

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Adrenaline given intravenously or subcutaneously to normal human subjects effects an increase in blood-sugar and blood-lactic acid (Cori & Buchwald, 1930; Loeb, Reeves & Glasier, 1931), and a fall in serum potassium (Castleden, 1938; Keys, 1938). In animals intravenous adrenaline causes a fall in muscle glycogen (Cori, 1931). This also occurs in man (Hildes, Sherlock & Walshe, 1949).

In their studies on muscle-blood flow, Allen, Barcroft & Edholm (1946) described a technique for infusing adrenaline intra-arterially in man. It seemed to us that adrenaline given by this route might produce an even greater depletion of muscle glycogen in the infused leg than when given intravenously, although when given intra-arterially it does not have its usual systemic effects on the cardiovascular system.

MATERIAL AND METHODS

Material. Clinical notes on the twelve patients used in this investigation are given in the Appendix. They were all males ranging in years from 27 to 67, and when the observations were made they were, on the whole, free of symptoms and on a full hospital diet. None had any known defect of carbohydrate metabolism. The observations were made after a simple explanation of the procedure to the patient.

Experimental procedure. All observations were made in the morning after a 12 hr. fast in bed. 200 mg. sodium amytal was given about 1 hr. before the observations began. In three cases this mild sedative was omitted (Table 1). Adrenaline was infused into one femoral artery for a period of ½–¾ hr. This was preceded and followed by control periods of about 45 min. During the preliminary control period, in all but three cases, the femoral vein on the same side was catheterized for sampling. Frequent measurements of the blood pressure and pulse rate were made throughout the experiment, and changes in colour of the feet were noted.

Samples of capillary, brachial venous and femoral venous blood were taken at 10–15 min. intervals. Glucose was estimated in all samples, lactate and serum potassium in the venous samples. In most cases the glycogen content of the gastrocnemius muscle on the side of the infusion was

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estimated before, during and after the infusion. The methods used are described in more detail in view of some of the difficulties encountered.

Intra-arterial infusion. The femoral artery was chosen in preference to the brachial artery because of the ease of gastrocnemius muscle biopsy. The site selected for arterial puncture was 1–2 in. below the inguinal ligament. With the patient supine, the skin and subcutaneous tissues were anaesthetized with 2% procaine solution. A fine-bore needle (gauge 24) could then be introduced into the artery without difficulty and without discomfort to the patient. The infusion was maintained at a constant rate, using a motor-driven 50 c.c. syringe. This apparatus was modified from the model used by Allen *et al.* (1946). This delivers, against arterial pressure, at a constant rate ranging from 1 to 5 c.c. a minute. The rate of the infusion can be accurately and finely adjusted.

The arterial needle was fixed by dental wax to one end of a convenient length of transparent plastic tubing (Telcothene), which was connected through a 3-way tap to the nozzle of the syringe. The whole was filled with the diluted adrenaline solution and the syringe placed in position. The needle was then introduced into the femoral artery, held in place by adhesive strapping and the motor started. The patency of the needle could be checked at any time by momentarily turning the tap when arterial blood appeared in the end of the transparent tubing. By this arrangement it was possible to maintain an infusion for 45 min. without recharging the syringe. This prevents a needle of this bore blocking, as it tends to do when introduced into the artery attached to a small syringe in the usual way and then connected to rubber transfusion tubing.

In two cases, after about 10 min. of infusion, when the local anaesthesia had diminished, the patients complained of a numb burning sensation in the distribution of the medial cutaneous branch of the femoral nerve. In these cases the experiment was discontinued and the pain disappeared in about 15 min.

Femoral vein sampling. A small incision was made through local anaesthesia 1½ in. below and lateral to the pubic spine and the saphenous vein located. Through this vein a short length of sterile plastic tubing was introduced approximately 6 in. into the femoral vein for sampling. Between samples the tubing was kept patent by a slow saline infusion.

Muscle-glycogen estimation. Muscle samples of 20–100 mg. in weight were taken by biopsy of the ipsilateral gastrocnemius under local anaesthesia (Hildes, Sherlock & Walshe, 1949). Samples were taken immediately before the infusion, just before its termination and ½–1 hr. after the infusion ended. Duplicate, and in some cases, triplicate samples were analysed for glycogen content (Hildes *et al.* 1949).

Blood-sugar estimations were carried out in duplicate, by the method of Haslewood & Strookman (1939), using 0·05 ml. samples in the case of capillary blood and 0·2 ml. samples for venous blood.

Blood-lactic acid was estimated by the method of Long (1946).

Serum potassium was estimated by the method of King, Haslewood, Delory & Beall (1942).

Dosage and preparation of adrenaline. Ampoules of 1:1000 adrenaline (Allen and Hanbury) were suitably diluted in normal saline containing 50 mg. ascorbic acid per 100 ml. (Cori, Fisher & Cori, 1935). The infusion was at a constant rate, but the dosage in the twelve subjects ranged between 0·032 and 0·12 μg./kg. body wt./min. (Table 1). This covers the range of intravenous dosage used by other workers (Cori & Buchwald, 1930; Loeb *et al.* 1931, and others) and approximates to the intra-arterial dose used by Allen *et al.* (1946). These workers infused adrenaline for only 10–12 min. The maximal effect of intravenous adrenaline on muscle glycogen in man probably occurs at 30 min. (Hildes *et al.* 1949), and we decided to infuse for approximately this time.

RESULTS

The results of ten experiments are summarized in Table 1, and the details of one of the experiments are shown in Table 2.

Effects on the cardiovascular system. Blood pressure and pulse rate were taken at frequent intervals during the experiment (Table 1). In one case blood-pressure readings were omitted because of difficulty in venous sampling from

TABLE 1. Effects of intra-arterial adrenaline in ten cases

Case no.	Adrenaline (μg./kg./min.)	Time of infusion (min.)	Systolic B.P. (max. rise mm. Hg)	Pulse rate (max. rise beats/min.)	Maximum change in blood sugar (mg./100 ml.)*			Maximum change in blood lactic acid (mg./100 ml.)*		Muscle glycogen (g./100 g.)		
					Capillary	Brachial venous	Femoral venous	Brachial venous	Femoral venous	Control	Change‡ affected adrenaline	30 min. after infusion
1	0·110	46	4	0	+16	+8	—	−0·2	—	—	—	—
4†	0·084	45	4	4	−6	−8	−3	−2·0	+0·5	—	—	—
5†	0·032	38	4	0	+8	—	—	—	—	1·98	−0·14	1·78
6†	0·066	35	0	0	—	−1	+15	+1·3	+1·4	—	—	—
7	0·070	28	12	12	+25	—	—	—	—	1·77	+0·11	1·79
8†	0·100	24	8	0	0	—	+4	−0·3	+7·5	3·03	−0·02	3·07
9†	0·100	23	2	8	+7	+1	+4	+1·3	+1·6	—	—	—
10†	0·120	24	8	12	+7	+7	+10	+1·0	+2·1	1·54	+0·06	1·50
11†	0·100	40	—	4	+2	+8	+2	+0·9	+0·9	1·32	+0·05	1·35
12†	0·096	42	10	20	+9	+14	—	+5·2	—	1·67	−0·05	1·68
Means	0·088	34	5	6	+8	+4	+6	+0·9	+2·3	1·89	+0·005	1·86
Mean values for intravenous adrenaline in 20 subjects (Hildes, Sherlock & Walshe, 1949)												
	0·081	60	32	20	+48	—	—	+8·7	—	—	−0·60	—

* Maximum change recorded during the infusion compared with the mean control value.

† Sodium amytal 200 mg. orally 1 hr. prior to observation.

‡ Change in muscle glycogen at end of the infusion compared with the control value. In case 5 a sample 15 min. after starting the adrenaline contained 1·95 g. glycogen per 100 g.

that arm. In no case was there any noteworthy change in blood pressure or pulse rate except in case 12 where the pulse increased 20 beats per min.

No measurements were made of skin temperature or muscle-blood flow, but alterations in skin vessel tone were assessed by comparing the skin colour of the feet. Blanching of the foot on the operated side was evident within 2½ min. of starting the infusion and was maintained throughout. When the infusion was stopped, there was a hyperaemic flush which came on slowly, and at first unevenly, to reach maximal intensity within 5 min. and then subsided to normal colour within 10 min.

TABLE 2. Intra-arterial adrenaline infusion. Case No. 11

	Blood-sugar (mg./100 ml.)			Blood-lactic acid (mg./100 ml.)		Serum potassium (mg./100 ml.)		
Time (min.)	Capillary	Brachial venous	Femoral venous	Brachial venous	Femoral venous	Brachial venous	Femoral venous	Muscle glycogen (g./100 g.)
				Preliminary control period				
0	93	85	85	4·5	4·8	—	22·4	—
15	—	—	—	—	—	—	—	1·32
30	96	84	87	5·8	8·0	20·7	20·0	—
			Adrenaline infusion 0·100 μg./kg./min. for 40 min.					
70	93	87	88	6·1	7·3	20·0	20·4	—
85	97	91	87	5·1	4·5	20·7	—	—
110	—	93	89	4·2	5·1	19·2	20·7	1·37
				Control period				
120	104	—	102	—	7·0	—	20·0	—
140	97	94	95	4·5	10·1	20·0	20·0	—
160	87	95	92	7·7	8·6	—	20·0	1·35

Facial pallor was noted in some cases but this was never very marked and was delayed in onset. When the infusion was stopped, there was no marked flushing of the face, although in one or two cases a slight flush was noted. This is in marked contrast with the immediate and striking pallor of the face seen when adrenaline is given intravenously, and the marked facial flushing when intravenous adrenaline is stopped.

Effect on blood sugar. The maximum changes in blood sugar above the fasting level are shown in Table 1. The mean of these changes was +8 mg./100 ml. in the case of capillary blood, +4 mg./100 ml. in brachial venous blood and +6 mg./100 ml. in femoral venous blood. There is no statistically significant difference between the mean value of capillary blood sugar before and the mean value during the adrenaline infusion (difference between the means = 7·1 mg./100 ml., 2 × standard error of the difference = 9·6).

However, in four cases one or other blood sample did show a rise in blood sugar of 14 mg. or more per 100 ml., the highest being 25 mg./100 ml. above the fasting level (Table 1). It is interesting to note that the high blood-sugar level in each of these four cases was delayed, in one 20 min. and in the others 30 min. after the start of the adrenaline infusion. Three of the subjects had not received sodium amytal. This substance given in a dose of about 50 mg./kg. body wt. is known to stabilize carbohydrate metabolism in animals. The dose of sodium amytal given to our cases was only of the order of 3·5 mg./kg. body wt. The slightly greater rise in blood-sugar of some patients that did not receive the drug is therefore considered fortuitous.

Effect on blood-lactic acid. Table 1 shows the changes in blood-lactic acid in both brachial and femoral venous blood. The values in the table show the maximum change that occurred in each case. The differences between the mean values before and during adrenaline infusion were 0·4 mg./100 ml. and 0·7 mg./100 ml. for brachial and femoral blood respectively. Neither of these differences is significant.

Effect on serum potassium. There was no significant change in serum potassium concentration either in brachial venous blood or femoral venous blood when adrenaline was infused into the femoral artery. In the case of brachial venous blood, the mean values before and during infusion were 20·5 and 20·1 mg./100 ml. respectively. The mean values for femoral blood were 19·2 and 18·3 mg./100 ml. before and during infusion.

Effect on muscle glycogen. Duplicate, and sometimes triplicate, samples were taken at each biopsy and analysed for glycogen (Good, Kramer & Somogyi, 1933; Hildes *et al.* 1949). Initially, some difficulty was encountered in obtaining accurate duplicate checks. With practice, in particular regard to muscle sampling, this difficulty was overcome to the extent that a deviation of the order of 16% from the fasting level could be regarded as significant ($P < 0{\cdot}05$). Only the muscle glycogens estimated since accepting this standard of analysis have been included in Tables 1 and 2. The changes in muscle glycogen produced by intra-arterial adrenaline were studied in six cases. The mean values and standard deviation during and after the infusion, expressed as a percentage of the fasting level, were found to be 101% (S.D. 7·5) and 98·6% (S.D. 7·5) respectively.

DISCUSSION

Adrenaline given intravenously results in a breakdown of liver glycogen to glucose with a rise in blood-sugar values. There is also glycogenolysis in muscle with lactacidaemia. If adrenaline produces a direct effect on muscle glycogen, infusion into an artery should result in a very conspicuous fall in the muscle glycogen of the related limb with a subsequent rise in blood-lactic-acid values. However, the present experiments show that intra-arterial adrenaline does not result in any significant change in the glycogen content of the muscle of the limb infused. Moreover, the lactic-acid content of the venous blood from that limb is not increased. These observations suggest that adrenaline does not have a direct effect on muscle glycogen. Adrenaline is destroyed by tissues very rapidly and remains for only a short time in the blood stream (Weinstein & Manning, 1937; Richter & Tingey, 1939). If given into an artery it is probably inactivated in the limb tissues and, provided the dose is not excessive, general effects should be minimal. This is borne out by the present results. Intra-arterial adrenaline produced little rise in the systemic blood sugar and in those cases in which a slight increase was noted it was delayed 20–30 min. after starting the infusion.

We have found no other experiments reported that bear directly on this point. Griffith, Lockwood & Loomis (1946), studying the effects of intra-arterial adrenaline on sugar retention by the leg tissues of anaesthetized cats, found that doses which cause vasodilatation may cause a twofold increase in lactate output. This implies an increased breakdown of muscle glycogen. However, muscle glycogen was not estimated. This data on lactate output was considered inconclusive, but the trend was in agreement with the findings of Cori *et al.* (1935). The findings of Cori and his collaborators are based on experiments with intravenous adrenaline. In a subsequent paper, Griffith, Omachi, Lockwood & Loomis (1947) found much higher lactate output using intravenous adrenaline. Borysiewicz (1930) injected adrenaline into the femoral artery of dogs and found a normal glycaemic response but no change in blood pressure. Baudouin, Bénard, Lewin & Sallet (1936), also in dogs, using the infusion technique, report that to produce the same glycaemic effect by intra-femoral arterial injection, twice to four times the intravenous dose is necessary. In neither of these papers were the blood-lactic acid and muscle-glycogen values recorded. A dose of adrenaline comparable with that used in the present work infused into the aorta of rats resulted in a fall in the glycogen content of the related muscle and a rise in blood-lactic acid (Sherlock, 1949). None of the above observations is readily comparable with the results of the present study—the species used, the type of anaesthesia and the technique of administration being very different.

Adrenaline is known to stimulate the anterior pituitary to release adrenocorticotropic hormone (Long, 1947). It might be postulated that the changes in muscle occurred secondarily to pituitary stimulation. However, adrenaline given intravenously to hypophysectomized animals produces all its usual effects on carbohydrate metabolism (Russell & Cori, 1937), so this does not seem to be the case. The mechanism by which adrenaline causes muscle glycogenolysis in man is still obscure.

Allen *et al.* (1946) injected half our dose of adrenaline intra-arterially into normal man during 5 or 24 min. Conspicuous changes occurred in muscle-blood flow in the limb infused but without significant change in heart rate or general blood pressure. The effect on muscle-blood flow in anaesthetized cats varies with the dose (Griffith *et al.* 1946; Griffith *et al.* 1947); small doses produce vasodilatation and larger doses vasoconstriction. In the present experiments, although the intra-arterial adrenaline did not disturb muscle glycogen there was pallor of the skin of the limb perfused. This indicates constriction of skin vessels. Again there was no change in heart rate or blood pressure. Adrenaline therefore does appear to act directly on blood vessels, constricting those of the skin and dilating those of the muscle.

SUMMARY

1. Adrenaline was infused into the femoral arteries of ten normal men in an average dose of 0·088 μg./kg. body wt./min. Measurements of general blood pressure and pulse rate, skin colour, blood-sugar, blood-lactic acid serum potassium and muscle glycogen were made.

2. Blood pressure and pulse rate showed only trivial changes. Pallor of the skin of the infused limb was produced and this remained throughout the period of infusion.

3. Blood-sugar, blood-lactic acid and serum potassium showed no significant change either in a systemic vein or in the femoral vein of the limb perfused.

4. The muscle glycogen content of the gastrocnemius of the limb perfused did not change.

5. Adrenaline introduced intra-arterially in man apparently has no direct glycogenolytic effect on muscle. It is probably rapidly destroyed or changed in the limb tissues and produces no general effects on carbohydrate metabolism.

We are indebted to Prof. F. G. Young for helpful criticisms and to Miss Z. N. Taylor for technical assistance.

APPENDIX

	Case	Age	Wt. (kg.)	Diagnosis
1.	E.N.	34	50	Bronchial asthma
2.	J.C.	69	60	Chronic bronchitis
3.	W.C.	54	47	Convalescent bronchopneumonia
4.	H.B.	58	64	Convalescent gastric ulcer
5.	A.T.	67	55	Convalescent gastric ulcer
6.	W.P.	63	54	Amytrophic lateral sclerosis
7.	J.G.	49	65	Convalescent duodenal ulcer
8.	J.H.	59	50	Convalescent gastric ulcer
9.	G.P.	50	58	Gastric ulcer and eczema of hands
10.	F.P.	27	55	Duodenal ulcer
11.	G.S.	67	67	Convalescent duodenal ulcer
12.	J.C.	46	58	Convalescent duodenal ulcer

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THE EFFECT OF TEMPERATURE ON THE ELECTRICAL ACTIVITY OF THE GIANT AXON OF THE SQUID

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The effect of temperature on the electrical activity of nerve and muscle has been studied intermittently since the time of Bernstein (1902). Among the most important investigations are those of Lucas (1908), Verzár (1912), Adrian (1921), Gasser (1931), Bremer & Titeca (1933, 1946), Auger & Fessard (1936), Schoepfle & Erlanger (1941), Cardot & Arvanitaki (1941), Lorente de Nó (1947), Tasaki & Fujita (1948) and Lundberg (1948). There appears to be general agreement that the resting potential has a low temperature coefficient, but the evidence concerning the effect of temperature on spike amplitude is conflicting. The experiments of Gasser (1931) suggest that the temperature coefficient of the spike is large and positive, while those of Schoepfle & Erlanger (1941) indicate that it is small and negative. Recent work on the giant axon of the squid suggests that the effect of temperature should be examined with this preparation. In particular, it is important to know whether the characteristic relation between action potential and resting potential holds over a wide range of temperature. The use of an internal recording electrode facilitates this study, since membrane potentials can be measured directly and are independent of the amount of external short circuiting. Another important advantage of this technique is that the temperature of the preparation can be altered rapidly by changing the sea water in which the axon is immersed.

METHOD

The experiments were made on the largest axon (diameter 500–700μ) of the stellar nerve of *Loligo forbesi*. The mounting of the fibre, insertion of the microelectrode and the method of recording action potentials and rates of potential change have been described by Hodgkin & Huxley (1945) and Hodgkin & Katz (1949).

The temperature was varied by replacing the sea water-bath around the axon (about 100 c.c.) and was read on a thermometer inserted close to the nerve. The effects of temperature change on the spike and resting potential were established without noticeable lag, provided that the temperature was not raised much above 35° C., at which point the axons tended to fail progressively. It was, therefore, possible to obtain a continuous range of measurement by allowing the bath to warm or cool gradually towards room temperature. A temperature run could be made in about 15 min., and all effects were found to be completely reversible provided that the temperature did not exceed 35° C. The lowest temperature which could be achieved with the present technique was about 1° C. By using a sea water-ice mixture the chamber was brought, on one occasion, to -1° C., but this was not a satisfactory procedure because it involved the risk of damaging the fibre by mechanical or osmotic effects.

The action potential records were measured after projection on to a calibration grid, so that non-linear distortions of the co-ordinate system were corrected. The size of the action potential could be measured to less than 1 mV. In the present investigation the arrangement of the input system was such that the recording system responded with an exponential lag of 11 μsec. This was large enough to reduce the spike by 2 or 3 % at temperatures above 30° C. but this error was insignificant compared with the observed effects. The maximum rates of rise and fall were measured by graphical or electrical differentiation as described elsewhere (Hodgkin & Katz, 1949). The differentiating circuit had a time constant of 13 μsec. and this probably introduced an error of about 10 % at the highest temperatures. The graphical method was liable to rather larger errors which increased with the curvature of the records and was only applied to measurements at low temperature.

The errors involved in the measurement of the resting potential have been discussed by Curtis & Cole (1942) and Hodgkin & Huxley (1945). The values given in this paper were corrected for the potential difference between the two electrodes in sea water, but no allowance has been made for the diffusion potential at the sea water-axoplasm junction of the internal electrode. The true values of the membrane potential are likely to be 5–15 mV. higher than those recorded experimentally. The individual measurements depended upon the stability of electrode and amplifier system and were accurate only to within about 2 mV. A control run was made to determine whether the electrode potential difference was affected by changes of temperature in the sea water-bath. The microelectrode should not have been affected as it made contact with a chlorided silver wire well outside the bath, but the external electrode (which was inserted in the perspex wall of the chamber) underwent slow temperature changes during the course of an experiment. The error due to this cause was always less than 2 mV. and its effect was reduced by checking the resting potential immediately before and after each temperature run. Finally, a small systematic error may have been introduced by the effect of temperature on the sea water-axoplasm junction. The magnitude of this error cannot easily be estimated, but it is not likely to exceed 1 or 2 mV. over the whole temperature range employed.

RESULTS

Resting potential

Temperature has little effect on the resting potential, as may be seen from the individual experiment plotted in Fig. 1, curve *B*. The resting potential is practically constant or diminishes very slightly as the temperature is raised from 3 to 20° C. At higher temperatures it falls appreciably and is reduced by 10–15 mV. at 35° C.

Similar results were obtained in eleven other experiments which are summarized in Table 1A and Fig. 2. There was considerable variation in the absolute magnitude of the spike and resting potential, but the temperature effect was quite consistent. The variation may have arisen from the fact that some of the axons had been used for other work before the temperature runs were started and therefore showed reduced membrane potentials.

To obtain a fair estimate of the consistency of the temperature effect the results of each experiment were scaled so that the resting potentials at 20° C. coincided with the mean at this temperature (i.e. 47 mV.). The scaled values have been plotted in Fig. 2, curve *B*, and the means at seven temperatures are given in Table 1C. The results show that the resting potential is almost independent of temperature between 2 and 20° C. Above this temperature it diminishes and at 35° C. it is reduced by about 10 mV.

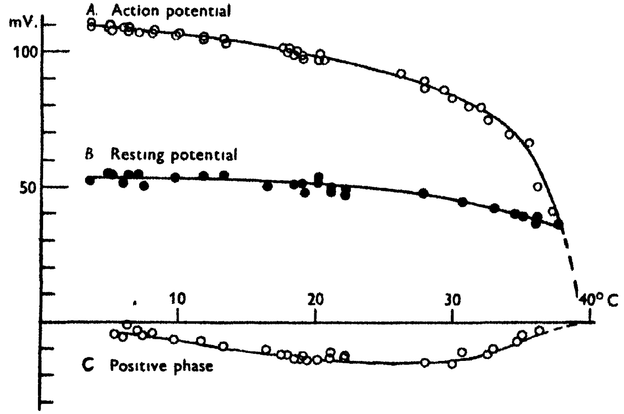

Fig. 1. Effect of temperature on the magnitudes of *A*, action potential, *B*, resting potential and *C*, positive phase.

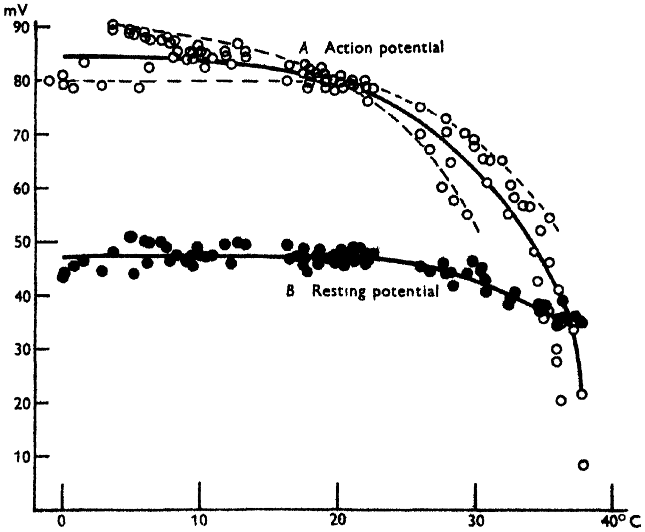

Fig. 2. Effect of temperature on action potential and resting potential, showing the results of twelve experiments. The full lines represent an average experiment, the broken lines show extreme variations in the effect of temperature on the action potential.

Table 1. Effect of temperature on resting and action potentials

A. Resting potential (mV.)

	Temperature (° C.)							
Exp.	0	5	10	15	20	25	30	35
1	—	—	46	46	46	—	—	—
2	—	—	—	—	48	46	43·5	—
3	—	54	53	52·5	51	49	45	40
4	—	—	—	49	48·5	47	45·5	40·5
5	—	50	50	50	49	47·5	45·5	40·5
6	—	—	51	51	51	—	—	—
7	—	44·5	44·5	44·5	45	—	—	—
8	44·5	44·5	45	45	45	—	—	—
9	45·5	45·5	46	46	46	—	—	—
10	—	—	—	—	44·5	44·5	43·5	34
11	—	—	49	49	48	45	41	—
12	—	—	—	—	45	43·5	40·5	36

B. Action potential (mV.)

	Temperature (° C.)							
Exp.	0	5	10	15	20	25	30	35
1	—	—	76·5	74·5	72	—	—	—
2	—	—	—	—	66·5	60	40·5	—
3	—	109	106	103	98	92·5	83	66
4	—	—	—	88	84·5	79·5	71·5	46
5	—	89	86·5	83·5	80·5	74·5	61·5	35
6	—	—	86·5	84·5	83	—	—	—
7	—	—	77	75·5	73·5	—	—	—
8	84·5	83·5	82·5	82	81	—	—	—
9	79·5	79	79	79	79	—	—	—
10	—	—	—	—	72	68	61	47
11	—	—	—	94	88	80	66	—
12	—	—	—	—	82	78	69	50

C. Mean values, computed after scaling all experiments to their common mean at 20° C. Numbers of experiments shown in brackets

	Temperature (° C.)						
	5	10	15	20	25	30	35
Resting potential (mV.)	47·5(5)	47·5(8)	47·5(9)	47(12)	45·5(7)	43(7)	38(5)
Action potential (mV.)	85(4)	83·5(7)	82·5(9)	80(12)	74·5(7)	62·6(7)	46·5(5)
Positive phase (mV.)	4·5(4)	7(6)	10(8)	12(10)	12(6)	10(6)	6(4)
Max. rate of rise (V. × sec.$^{-1}$)	148(4)	254(8)	359(8)	500(8)	533(2)	558(2)	—
Max. rate of fall (V. × sec.$^{-1}$)	39(4)	91(8)	165(8)	301(8)	384(2)	455(2)	—

Note. No allowance has been made for the sea water-axoplasm junction potential (see Method).

Action potential

The temperature effect is illustrated by Figs. 3 and 4, in which several records have been superimposed. The most obvious result is the slowing, at low temperature, of the action potential wave, in particular of its declining phase. But perhaps equally remarkable is the fact that the amplitude of the spike is not altered greatly except at temperatures above 30° C. in which region it falls rapidly (Fig. 5). Above 35° C. the spike became very small and conduction failed in the vicinity of 40° C. The spike reappeared when the axon was cooled and increased rapidly as the temperature fell to 30° C. (Fig. 5). The temperature at which heat block occurred and the degree of recovery from this effect showed considerable variation. Fresh fibres were able to conduct impulses of reduced size at 37–38° C., but after two or three temperature runs the response diminished and recovery became incomplete. In the low temperature range the experiments were not pursued far enough to decide whether a cold block could be produced without freezing the axon. At -1° C. the axon was capable of conducting a spike of the same size as at 20° C. (Fig. 2*A*) and it seems unlikely that a fresh squid nerve can be blocked above the freezing-point of sea water ($-1{\cdot}88^\circ$ C.).

The relation between action potential and temperature shown in Figs. 1*A* and 4 was confirmed in several other experiments which are summarized in Table 1B. To show the variability of the temperature effect the results were scaled in the same way as for the resting potential and are plotted in Fig. 2*A*.

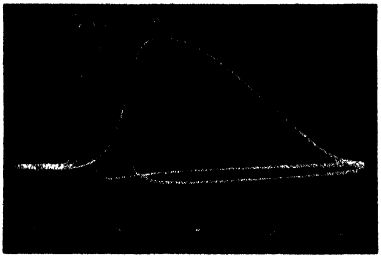

Fig. 3. Records of action potentials at three temperatures, superimposed on the same base-line and stimulus artefact. Temperatures and amplitudes, respectively: *A*, 32·5° C., 74·5 mV.; *B*, 18·5° C., 99 mV.; *C*, 5° C., 108·5 mV. Time marks: 1 msec.

The heavy line in Fig. 2*A* represents a mean curve, while the broken line illustrates the extreme cases which have been observed. The upper contour appeared to be characteristic of fresh axons, while the lower contour corresponded to axons which had been used for some time. Average values at seven temperatures are given in Table IC.

A characteristic property of the squid giant axon is the diphasic nature of its membrance action potential (Curtis & Cole, 1942). It differs in this respect from the monophasic spike of crustacean nerve (Hodgkin, 1938). At 20° C. the average value of the positive phase was 12 mV., although 14 mV. is probably a more representative value for fresh fibres. The effect of temperature is seen in curve *C*, Fig. 1, and mean values at seven temperatures are shown in Table 1C. The positive phase passes through a maximum at 20–25° C. At higher temperatures it is reduced in proportion to the height of the spike. But at low temperatures the positive deflexion is diminished while the spike is larger. In its general appearance the action potential of cooled squid nerve is similar to that of crustacean nerve.

The curves in Fig. 4 suggest that the rate of fall of the action potential has a higher temperature coefficient than the rate of rise. This possibility was examined in detail by the following methods.

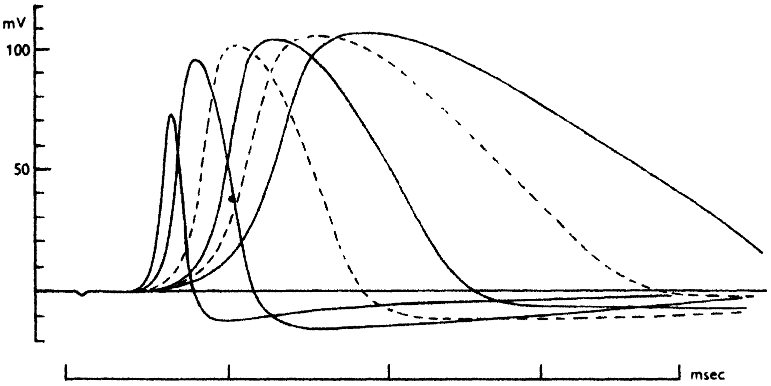

Fig. 4. Superimposed tracings of six spikes at the following temperatures: 32·5, 20·2, 13·3 (broken line), 9·8, 6·3 (broken line) and 3·6° C.

Fig. 5. Effect of high temperature on the squid giant axon. The temperature was progressively lowered from 38 to 20° C. The very small response at 37·8° C. was possibly due to a decrementing wave spreading beyond a block. The resting potential is shown by the distance between each base-line and the continuous horizontal line, the latter representing zero potential difference between the recording leads. *A*, 37·8° C.; *B*, 36° C.; *C*, 32·9° C.; *D*, 21·2° C.

(*a*) The *durations* of rising and falling phases of the negative spike were measured, taking a small initial deflexion (about 0·5 mV.), the peak and the crossing of the base-line as the three reference points. The result of a typical experiment is shown in Fig. 6 where the durations of rise and fall have been plotted on a logarithmic scale against temperature. The temperature coefficient of the falling phase is evidently greater than that of the ascending phase and the two curves cross at 20° C. The quotient Q_{10}, i.e. the inverse ratio of durations for a change of 10° C., was determined for three ranges, viz. 5–10, 10–20 and 20–30° C.; the results are listed in Table 2A.

(*b*) The effect of temperature on the absolute rates of rise and fall is illustrated by Fig. 7. Although there may be some electrical distortion in these records, it is clear that the rate of fall grows more rapidly with temperature than the rate of rise. Mean values for the maximum rates of rise and fall are included in Table 1C.

(*c*) Another way of measuring rates is to consider percentage rates instead of absolute rates. This has been done in Table 2B for the purpose of comparison with the results in Table 2A. By maximum percentage rate of rise is meant $(100/P_0)\,(dp/dt)_{\text{max.}}$, where P_0 is the amplitude of the spike, and $(dp/dt)_{\text{max.}}$ is the maximum rate of rise.

The results of these three methods are consistent and lend some support to the suggestion that different mechanisms are responsible for the rise and fall of

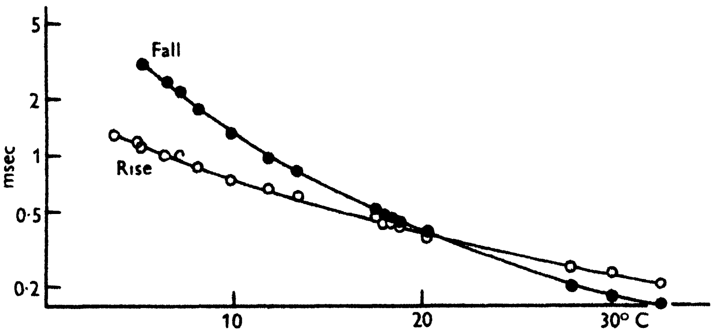

Fig. 6. Effect of temperature on rising phase (hollow circles) and falling phase (full circles) of the spike. Ordinates: durations, in msec., plotted on a logarithmic scale. Abscissae: temperature.

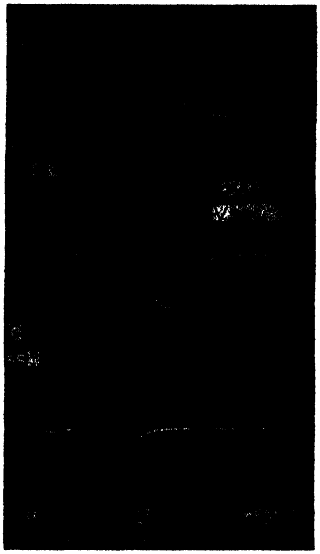

Fig. 7. Effect of temperature on the rates of rise and fall of the action potential. Records were obtained by electrical differentiation of the spike. The initial peak gives the maximum rate of rise, the trough shows the maximum rate of fall. The records show that the rate of fall has a higher temperature coefficient than the rate of rise. *A*, 12·5° C.; *B*, 18·8° C.; *C*, 33° C.

the action potential. In the squid nerve, at all events, it appears that the process underlying the restoration of the resting potential is characterized by its particularly high temperature coefficient.

TABLE 2. Effect of temperature on rise and fall of the action potential

A, Q_{10} values for the reciprocal duration of rising and falling phase. B, Q_{10} values for maximum percentage rates of rise and fall.

Exp. no.	Q_{10} of rise (° C.)			Q_{10} of fall (° C.)		
	5–10	10–20	20–30	5–10	10–20	20–30
A. 1	—	2·1	1·74	—	3·5	2·7
2	2·4	2·0	1·6	6·3	3·4	2·1
3	2·9	2·1	—	5·5	3·7	—
4	2·75	1·86	1·55	4·2	3·1	1·85
5	—	2·3	1·45	—	2·8	2·0
6	—	—	1·35	—	—	1·65
Mean	2·7	2·07	1·54	5·3	3·3	2·06
	Rate of rise (° C.)			Rate of fall (°C.)		
	5–10	10–20	20–30	5–10	10–20	20–30
B. 1 (E.)	—	1·95	—	—	2·79	—
2 (G.)	2·19	2·19	—	5·0	3·47	—
3 (E.)	2·2	1·95	—	4·6	3·8	—
4 (E.)	—	1·7	1·48	—	2·7	1·95
5 (G.)	2·37	—	—	4·28	—	—
6 (E.)	—	1·8	1·29	—	3·07	1·9
Mean	2·25	1·92	1·39	4·65	3·17	1·93

(E), electrical; (G), graphical differentiation.

DISCUSSION

The effect of temperature on bioelectric potentials has attracted considerable theoretical interest. Thus, Bernstein (1902) pointed out that the small positive temperature coefficient which he observed in frog's nerve and muscle was compatible with his 'potassium membrane' hypothesis and with the idea that the resting potential is due to an ion concentration potential. Theoretically the membrane potential should have been proportional to the absolute temperature. In an attempt to explain divergences from this relation Bernstein was forced to assume that the permeability varied with temperature, and that permeability to ions other than potassium increased markedly above 20° C. A similar argument might be applied to the present results. It has been suggested (Krogh, 1946) that the transfer of sodium through cell membranes is governed by a special process involving a transient reaction with membrane molecules rather than simple diffusion through ion pores. It therefore seems possible that sodium transfer through the membrane might have a higher temperature coefficient than the migration of potassium and chloride ions. In this case an explanation is provided for the lack of proportionality between membrane potential and absolute temperature and for the decline in potential at high temperatures.

As regards the relation between spike and resting potential the previous observations (Curtis & Cole, 1942; Hodgkin & Huxley, 1945), which had been made at room temperatures, have now been confirmed over the whole range from 2 to 25° C. At all these temperatures the spike substantially exceeds the resting potential. At higher temperatures the spike is depressed more than the resting potential, and depolarization is incomplete above 30–35° C. These results agree with those of Bremer & Titeca (1933, 1946) who worked with frog's sciatic nerve and observed that a small decline of resting potential at high temperatures was accompanied by a much greater diminution of the spike. But it is clear that the potential difference across the active membrane may change only slightly over a wide range of temperature, and this seems consistent with the idea of a concentration potential as suggested in the 'sodium hypothesis' (Hodgkin & Katz, 1949). It can be argued that the inverse relation between spike height and temperature is a consequence of the transient nature of the action potential. The processes underlying the rising and falling phases must necessarily have opposite effects on the membrane potential, and it is to be expected that a rise in temperature, which accelerates the falling phase more than the rising phase, should have the effect of reducing the height of the spike.

The factors which determine the 'physiological' range of temperatures in different types of nerve are not yet understood. The squid axon ceases to conduct at a temperature which is optimal for mammalian nerve, while isolated mammalian or tropical frog's nerves fail when cooled to a temperature at which the response of the squid axon reaches its maximum amplitude (Grützner, 1878; Garten & Sulze, 1913). Garten & Sulze (1913) have shown that there is considerable variation between different species of frogs, and that the behaviour of isolated nerves at low temperatures depends upon previous acclimatization of the animals. This may explain the difference between Gasser's (1931) observation that the action potential of frog nerve declined sharply below 15–20° C. and might fail at 5° C. and those of other workers (see Garten & Sulze, 1913), who were unable to block conduction in frog nerve above the freezing-point. Whatever the explanation of these differences, it is clear that considerable caution must be used in generalizing the conclusions of a temperature study made on one particular type of nerve. At the same time there is some reason to believe that the present results may be applicable to other tissues besides the squid axon. The most accurate experiments on frog nerve are probably those of Schoepfle & Erlanger (1941), who studied the monophasic action potentials of single fibres in a small branch of the peroneal nerve. According to the data obtained by these authors, cooling from *c.* 25 to *c.* 8° C. increases the spike amplitude by a factor of about 1·2, the duration of the rising phase by *c.* 1·6 and the duration of the falling phase by 2–7. These results are seen to be not unlike those described in the present paper.

SUMMARY

1. Resting and action potential of the squid giant axon were recorded, with an internal electrode, at temperatures between −1° and +40° C.

2. The resting potential was practically constant up to 20° C., its mean value being 47 mV. At higher temperature, the resting potential declined, dropping by about 10 mV. between 20 and 35° C.

3. The action potential diminished in amplitude as the temperature was raised. This change was slight from 2 to 20° C., the mean value falling from 85 to 80 mV. The decrease in spike height became very rapid above 35° C., and a reversible 'heat block' occurred between 35 and 40° C.

4. The positive phase of the membrane action potential was diminished at low and high temperatures and reached a maximum, of approximately 10–15 mV., at about 25° C.

5. The rate of decline of the spike had a considerably higher temperature coefficient than the rate of rise, the Q_{10} values between 10 and 20° C. being 2·0 and 3·2 for rise and fall respectively.

We are greatly indebted to the Director and staff of the Marine Biological Laboratory, Plymouth, for their hospitality and for the generous facilities placed at our disposal.

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THE INFLUENCE OF SUPRARENAL DEFICIENCY AND OF SYMPATHECTOMY ON CREATININE CLEARANCE IN CONSCIOUS DOGS

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The first series of experiments described in this communication was designed to detect any change in creatinine clearance which might occur in adrenalectomized dogs during the early stages of the development of suprarenal deficiency. Reduction in the rate of creatinine clearance during suprarenal deficiency has previously been reported solely as a terminal event.

Dietary balance experiments in dogs, such as those of Harrop, Soffer, Ellsworth & Trescher (1933), which were confirmed and extended by the work of Loeb, Atchley, Benedict, & Leland (1936) and Stahl, Loeb & Atchley (1936) showed that a loss of sodium into the urine, accompanied by chloride loss, was the outstanding characteristic of suprarenal deficiency. The function of the renal tubules during suprarenal deficiency has therefore been examined by many workers. Harrison & Darrow (1939) found that the primary change in dog kidneys during suprarenal deficiency was a failure of the renal tubules to maintain the necessary concentration differences between urine and plasma with respect to certain ions. These same workers reported that a decrease in glomerular filtration rate, which they deduced from studies of creatinine clearance, contributed only to the final stages of renal disfunction during advanced suprarenal deficiency.

Creatinine clearance rates have been measured in trained, conscious, adrenalectomized dogs, during the early development of suprarenal deficiency. The degree of suprarenal deficiency, and the sufficiency of replacement therapy, were estimated in terms of the plasma chloride levels. Since the final stages of suprarenal deficiency are known to be accompanied by a rise in the non-protein-nitrogen (N.P.N.) of the blood (Harrop *et al.* 1933), whole blood N.P.N. values were estimated; the absence of any marked rise in N.P.N. of the blood in the presence of falling plasma chloride values was regarded as indicative of but a moderate degree of suprarenal deficiency. No detailed study of renal blood flow was attempted, but an indication of the state of the circulation was obtained from determinations of systolic blood pressures, circulation times, and haematocrit values.

Young (1939) demonstrated that, in cats, complete degeneration of nerve fibres to the chromaffin tissue of the suprarenal glands resulted from section of the splanchnic nerves coupled with the removal of the sympathetic chains from the crura of the diaphragm to the level of L_4. In order both to exclude the possibility that changes in creatinine clearance similar to those of suprarenal deficiency might result from preganglionic denervation of these glands, and to provide a control series of dogs whose creatinine clearance rates were examined before and after operative periods of approximately similar duration, creatinine clearance rates were examined in conscious dogs before, and 5–14 weeks after, sympathectomy. The evidence presented by Klisiecki, Pickford, Rothschild & Verney (1933) showed conclusively that in normal unanaesthetized dogs, denervation of the renal pedicle had no effect on water excretion, nor on the normal control of water excretion. Rhoads, Van Slyke, Hiller & Alving (1934) confirmed and extended these observations, and showed that denervation of the kidney pedicle produced no consistent change in either blood flow or urea clearance in conscious dogs. A diuresis, reported by numerous observers, which has resulted from renal denervation, appears to be characteristic of acute traumatic experiments performed under anaesthesia. In the experiments described below, the renal ganglia and nerves of the renal pedicle were left intact; the sympathetic denervation was preganglionic.

METHODS

Bitches were perineotomized, and were subsequently trained for more than a year to withstand the relevant experimental procedures without physiological disturbance. They were then subjected to a series of mock clearance tests before the beginning of the experimental period proper.

Operative. The sympathetic chains were removed from the stellate ganglia in the thorax above, down to the pelvis (Lockett, 1949). Adrenalectomy was completed in two stages, one week apart by the lumbar approach.

Creatinine clearance tests. The diet and daily routine were kept constant throughout the experimental period: creatinine clearance tests began at 10.30 a.m. after an 18 hr. fast: water was available throughout the fasting and experimental periods; the dogs not infrequently drank during the clearance tests, producing wide spontaneous variations in the rate of urine flow. Intravenous injection of 2 g. creatinine in 20 ml. 0·9 % NaCl (sterile) was followed in 5–10 min. by the beginning of the first of three consecutive estimation periods each of 12–25 min. duration. Venous blood samples were withdrawn from a malleolar vein near the mid-point of each period, but the true plasma value for each mid-point was determined graphically.

Urine was collected by catheter at the beginning and end of each estimation period. After the collection of each urine sample the bladder was immediately washed out with 10 ml. sterile 0·9 % NaCl, to ensure that bladder drainage had been complete, see Table 1. In order that figures may be available for calculations relating surface area to creatinine clearance, the stem lengths for the five dogs, nos. 1–5, are given: 77, 73, 72, 76, 73 cm. respectively.

The creatinine content of plasma, urine, and bladder wash-out fluid collected from each period were estimated by Folin's method (1914).

TABLE 1. Determination of creatinine clearance

Outline of Procedure:

Dog 2. 25 October 1948. Eight weeks after sympathectomy.

10.25 a.m. 2 g. creatinine in 20 ml. 0·9 % NaCl, injected slowly into left malleolar vein.

10.32 a.m. Bladder emptied by catheter, and washed out.

11.4, 11.35, 11.45 a.m. Urine collected quantitatively from the bladder by catheter. The bladder was immediately washed out with 10 ml. 0·9 % NaCl, and the wash out saline was added to the measured urine sample, forming the 'mixed urine' for each estimation period.

10.47, 11.15, 11.32 a.m. Blood samples of 5 ml. withdrawn into 0·1 ml. heparin (Liquemin, Roche products) from the right malleolar vein.

	Urine volumes (ml.)			Creatinine concentrations/100 ml.				
				Urine		Plasma		
Period	Total urine	Mixed urine	Min. output 'v'	Mixed (estimated)	Original (calculated) U (g.)	Sample estimated (mg.)	Extrapolated for time mid-point P (mg.)	Creatinine clearance $\frac{Uv}{P}$
1	27·0	38·5	0·9	1·231 g.	1·75	26·4	26·0	51·96
2	13·3	24·6	0·63	0·609 g.	1·13	14·5	15·0	47·03
3	8·1	17·0	0·4	0·628 g.	1·32	11·4	10·9	48·45

The mean of duplicate estimations is given in each case.

Plasma chloride and non-protein nitrogen values were determined by the micromethods described by King (1947).

Circulation time, leg to leg. The injection of 2 ml. of 1 % Chicago blue into the right malleolar vein took 2 sec. Twenty-five sec. after the beginning of the injection the first blood sample was withdrawn from the left malleolar vein into heparin, and thereafter blood samples were withdrawn every 5 sec. in order to determine the first blood sample in which Chicago blue could be detected in the plasma.

Blood pressures. These were determined on sleeping dogs as systolic arterial pressures from carotid loops with the corresponding sinus denervated, Verney & Vogt (1938). The dogs were equipped with conditioned sleep reflexes.

RESULTS

Creatinine clearance rates in suprarenal deficiency

Adrenalectomized dogs were maintained in good health on whole gland extract for 4–6 weeks. During this period the creatinine clearance was determined at intervals of about 5 days, and regular estimations of the leg to leg circulation times, non-protein-nitrogen of whole blood, plasma chlorides, and haematocrit values were made. When the normal range of these variables had been established for the individual dogs, whole gland extract was withheld. Deoxycorticosterone acetate was administered in amountss ufficient to delay, but insufficient to prevent, the development of suprarenal deficiency, which was indicated by a steady decline in the plasma chloride levels determined daily. The creatinine clearance rate was determined during this degree of suprarenal deficiency. Whole gland extract was then administered until the plasma chloride values returned to the normal range, when the rate of creatinine clearance was again examined. This experiment was repeated three times on each of these dogs. Fig. 1 is a graphical summary of the sequence of observations on one of these dogs.

The results of all these experiments, which were completed in the first 11–14 weeks immediately after adrenalectomy, are summarized in Table 2. The upper row of figures listed for each dog are those obtained in the initial control period, during which the adrenalectomized dogs were maintained in good health on whole gland extract. The sub-adjacent rows of figures are the values obtained during the recovery periods following suprarenal deficiency, when whole gland extract had been used to restore the plasma chlorides to the normal range of

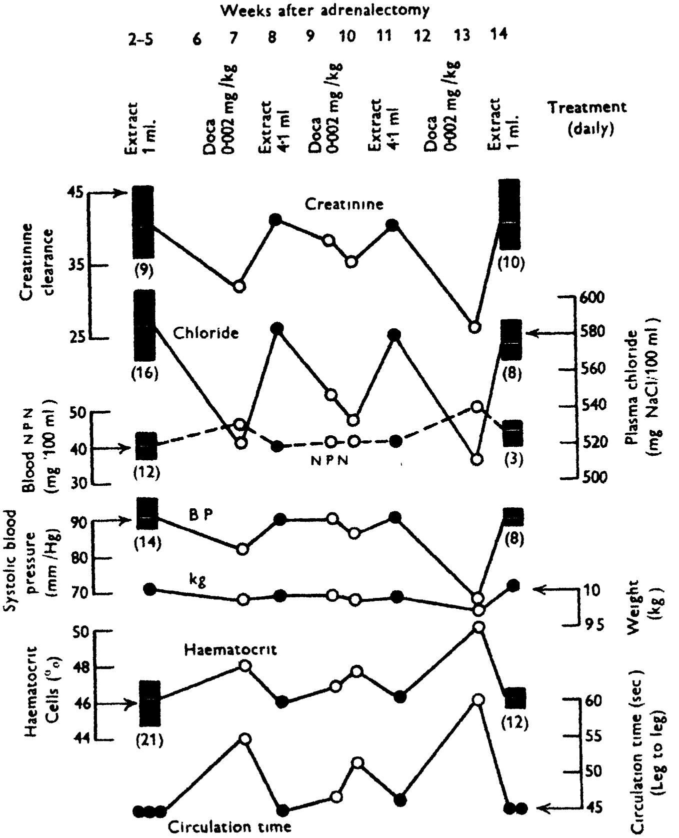

Fig. 1. A typical sequence of experiments performed on a single dog (dog 3). Rectangles represent the limits of the scatter of values of which the white bars are the means. Three periods of suprarenal deficiency alternate with periods of full substitution therapy. The values recorded during suprarenal deficiency are represented by open circles. Closed circles indicate observations made when the plasma chloride values had been restored to the normal range by extract therapy. The small weight of deoxycorticosterone acetate, 0·002 mg./kg. daily was sufficient only to delay the development of suprarenal deficiency. One ml. of extract = 75 g. suprarenal gland.

TABLE 2. Creatinine clearance during suprarenal deficiency

	Creatinine			Blood analysis			Circulation		
	Plasma clearance (ml./min.)	Plasma concentration (mg. %)	Urine flow (ml./min.)	Plasma chlorides (mg./100 ml.)	Non-protein N_2 (mg./100 ml.)	Haematocrit (%)	Time, leg to leg (sec.)	Systolic blood pressure (mm. Hg)	Weight (kg.)
3 *a*	40·7±1·1 (9)	30·6–7·8	0·1–1·7	588·2±2·9 (16)	41·1±0·6 (12)	45·8±0·4 (21)	45–50	91·3±0·6 (14)	10·0–10·2
b	39·9±1·2 (10)	31·2–6·6	0·2–1·3	574·7±2·7 (8)	42·9±0·6 (3)	46·0±0·4 (12)	45–50	89·2±0·2 (8)	9·9–10·1
	39·3±1·7 (3)	28·4–8·2	1·3–0·7	548	42	46·4	45–50	90	10·0
c	35·4±1·7 (3)	30·1–10·6	0·7–0·2	534	42	46·8	50–55	86	10·0
	32·3±1·1 (3)	32·3–12·8	1·1–0·3	522·5	47	48·0	55	82	9·9
	23·6±1·0 (3)	30·8–14·4	0·4–0·1	512	51	50·2	60	68	9·75
4 *a*	58·2±0·9 (9)	28·6–6·8	0·2–3·1	589·8±3·5 (28)	30·9±0·6 (16)	46·2±0·6 (27)	40–45	116·5±0·8 (16)	9·1–9·2
b	56·7±1·1 (9)	27·9–7·7	0·1–2·1	580·4±0·9 (13)	31·3±0·9 (3)	47·1±0·8 (7)	40–50	113·0±0·4 (17)	9·1
	45·7±3·3 (3)	28·2–11·1	1·1–2·1	539	32	49·2	45–50	110	9·1
c	36·6±1·2 (4)	28·7–6·6	0·9–0·6	528	34	50·0	50–55	96	Not weighed
	30·6±1·2 (3)	27·3–12·8	0·5–1·0	519	44	51·3	60	74	8·9
5 *a*	39·1±0·9 (9)	33·6–7·9	0·2–1·6	572±1·9 (19)	24·8±0·6 (8)	46·4±0·4 (14)	40	103·8±0·4 (9)	7·8–8·0
b	38·4±1·0 (6)	36·4–8·4	0·1–1·3	570±1·7 (9)	24·7±0·7 (4)	46·1±0·9 (7)	40	104·6±0·3 (6)	8·0–8·1
	37·4±2·6 (3)	32·3–10·8	0·7–1·2	543	23	47·0	40–45	102	7·9
c	33·8±1·9 (3)	31·8–11·7	0·6–0·2	532	26	47·8	45	90	7·85
	26·2±0·7 (3)	33·2–14·3	0·5–0·1	520	30	49·9	50	81	7·7

a, These figures are those from the initial control period. *b*, these figures are those from recovery periods after suprarenal deficiency. Throughout *a* and *b* dogs received full substitution therapy. *c*, The figures listed apply to periods of suprarenal deficiency. [Column 1. Mean ± standard error (Number of observations).]

values. Comparisons of these corresponding sets of figures show, first, that irreversible change in the creatinine clearance never resulted from the recurrent periods of moderate suprarenal deficiency; and, secondly, these observations establish the scatter of values normal, under the conditions of the tests, for each individual adrenalectomized dog in receipt of full substitution therapy.

The subsequent figures listed for each dog relate to the periods of moderate suprarenal deficiency. Reference to Table 2 shows that the range of creatinine plasma concentration studied, and the rates of urine flow recorded during suprarenal deficiency were throughout comparable with those examined during the control periods. The decreases in plasma chloride concentration and in creatinine clearance value as suprarenal deficiency progressed were accompanied by haemo-concentration and a decline in the weights of the animals: the systolic blood pressures also decreased, and the leg to leg circulation times increased.

Sympathectomy and creatinine clearance

The creatinine clearance values obtained from three dogs, 5–14 weeks after the completion of sympathectomy, have been compared with those obtained during a control preoperative period. The clearance tests were performed under the rigidly controlled conditions described above under 'methods'.

TABLE 3. Creatinine clearances before and after recovery from sympathectomy

Dog	Plasma cleared (ml./min.)	Range Urine vol. (ml./min.)	Range Plasma concentration (mg./100 ml.)	Systolic blood pressure (mm. Hg)	Weight (kg.)
I	75·7 ± 3·4 (6)	0·10–2·13	22·6–8·7	129·1 ± 0·24 (18)	9·0–9·1
	73·8 ± 2·7 (6)	0·43–3·4	21·8–10·1	125·4 ± 0·72 (8)	9·1–9·2
II	45·8 ± 2·0 (9)	0·10–1·33	26·4–5·7	116·8 ± 0·32 (15)	11·7–11·8
	44·1 ± 1·6 (9)	0·14–2·43	30·1–8·2	114·1 ± 0·68 (6)	11·8–11·9
III	40·5 ± 0·6 (9)	0·09–2·00	29·4–6·0	104·9 ± 0·21 (16)	10·1–10·0
	40·3 ± 1·2 (9)	0·11–1·46	34·2–6·4	100·2 ± 0·56 (18)	10·0

Pre-operative values for each dog are given by the upper rows of figures. The lower sets of figures show the corresponding values obtained 5–14 weeks after the completion of sympathectomy.

The collected results of clearance tests on three dogs before and after sympathectomy are shown in Table 3. From Table 3 it can be seen that no important change in weight resulted from operations, and that the ranges of plasma creatinine concentration and minute volume of urine studied before and after operation were comparable, and that the resting systolic blood pressures of the three dogs, during the interval occupied by the post-operative clearance tests, were only a few mm. Hg below those recorded during the pre-operative period. Table 3 also shows clearly that no significant alteration in creatinine clearance resulted from removal of the sympathetic chains.

Remarkably stable creatinine clearance values were obtained from these trained dogs throughout both sections of this work, but to achieve such constancy the experimental conditions had to be very meticulously standardized.

DISCUSSION

From the evidence presented in Table 2 it was evident that the reduction in creatinine clearance produced by moderate suprarenal deficiency was accompanied by circulatory changes. The prolonged circulation time, the fall in systolic blood pressure, and the increased viscosity of the blood which may be expected to have resulted from haemoconcentration, should probably be regarded as the initial changes of that profound circulatory collapse characteristic of the terminal stages of suprarenal deficiency. The decline in weight during advancing suprarenal deficiency must be related in part to the reduced food intake characteristic of such deficiency, but, since the weight loss was frequently very rapid, should also be regarded as part of a general decrease in water content of which haemoconcentration was but an example. The very small corrections that might be applied to the creatinine clearance values for this loss of weight, by reason of the probable effect on surface area as calculated from the Cowgill & Drabkin (1927) formula for dogs, are quite insufficient to account for the reduced creatinine clearance values recorded.

A fall in blood pressure may be expected to decrease the rate of glomerular filtration. That this is not the only cause for the reduced creatinine clearance values recorded during suprarenal deficiency is indicated by the results collected in Table 2. In the early stages of suprarenal deficiency, reduced creatinine clearance values were recorded in the absence of any significant alteration in systolic blood pressure. Information about the mean arterial blood pressure is not available. Lassen & Husfeldt (1934) showed in man, by the administration of spinal anaesthetics without ephedrine premedication, that a fall of 20 mm. Hg in the systolic blood pressure produced a 14 % reduction in the rate of creatinine clearance. Dog 3 showed a 40 % reduction, and dog 5 a 33 % reduction in the rate of creatinine clearance for a 200 mm. Hg fall in systolic blood pressure and dog 4 a 45 % reduction for a 40 mm. Hg decline in systolic pressure. Other factors must therefore be considered.

An increase in circulation time through the kidneys, such as has been recorded in the leg to leg observations of circulation time, and an increase in the viscosity and colloid content of the blood which may be expected to have resulted from haemoconcentration, would reduce the effective force available for glomerular filtration.

SUMMARY

1. Progressive decrease in the rate of creatinine clearance was observed in three adrenalectomized bitches during the early stages of the development of suprarenal deficiency. The latter was indicated by a decline in the daily plasma

chloride values. The decreased creatinine clearances were accompanied by reduced systolic blood pressures, by haemoconcentration, and by increase in the leg to leg circulation times, in the absence of any significant rise in N.P.N. (Table 2).

2. No alteration in the rate of creatinine clearance resulted in three bitches, from complete removal of the sympathetic chains from the stellate ganglion above to the pelvis below. Creatinine clearance rates were examined, and found within the normal range, from the fifth to the fourteenth post-operative weeks; resting systolic blood pressures had already returned to pre-operative levels (Table 3).

3. To establish the above findings the normal day to day scatter had to be determined for each of five bitches over a period of weeks. These bitches, which had been adrenalectomized, were maintained in good health by treatment with suprarenal extract of whole gland throughout these control periods. The coefficients of variation for creatinine clearance varied from 6 to 11 %; for plasma chloride from 1·8 to 3·1 %; for N.P.N. of whole blood from 5 to 7 %, and for systolic blood pressure from 1·1 to 2·8 %.

Prof. E. C. Kendall (Mayo Clinic) very kindly supplied the whole gland extract used in these experiments. A part of the expense of this work was defrayed by a grant from the Medical Research Council.

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PRESSOR RESPONSES TO ELECTRICAL STIMULATION OF THE CAROTID SINUS NERVE IN CATS

By E. NEIL and C. R. M. REDWOOD

Department of Physiology, School of Medicine Leeds

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(*Received* 26 *February* 1948)

Many authors (Hering, 1927; Koch 1931; Heymans, Bouckaert & Regniers, 1933; Schweitzer, 1937) describe the response of the carotid sinus nerve receptors to change of pressure in the isolated carotid sinus, and show clearly that these nerve endings act as baroceptors so that a rise of intrasinusal pressure causes, reflexly, a fall of systemic blood pressure. Action potential studies by Bronk & Stella (1932) support this view: a rise of pressure in the sinus is attended by an increased frequency of impulses in the afferent fibres of the carotid sinus nerve.

It is generally agreed that the baroceptors are stimulated only by a rise of pressure in the sinus, and evidence exists that there is a threshold pressure (20–80 mm.Hg in different species) below which activation of the baroceptors does not occur (Koch, 1931; Schweitzer, 1937).

In view of the large number of observations on carotid sinus function, it is perhaps surprising that there are only few observations with reference to the effects of electrical stimulation of the nerve trunk. The development of the rectangular wave electronic stimulator permits a more detailed investigation of this problem, and the present paper gives results obtained by the use of this device and of a conventional induction coil. Some of our observations have previously been reported (Neil, Redwood & Schweitzer, 1948).

METHODS

Forty-five cats were used in these experiments. The animals were anaesthetized with chloralose (0·08–0·1 g./kg. body weight, intravenously), chloral hydrate (0·15–0·2 g./kg. body weight, intravenously) or nembutal (Pentobarbitone sodium) (35–40 mg./kg. body weight, intraperitoneally). In other experiments cats were decerebrated under light ether anaesthesia; at least 60 min. were allowed for the effects of the volatile anaesthetic to pass off before experimentation was started. In a number of decerebrate cats either chloralose or nembutal was injected at a later stage of the experiments.

The carotid sinus nerve was prepared for stimulation using an approach similar to that described by Von Euler, Liljestrand & Zotterman (1939). The glossopharyngeal nerve was dissected free and the carotid sinus nerve carefully bared from its junction with the glossopharyngeal nerve down to the tissue lying in the region of the common carotid artery bifurcation.

Fine shielded silver wire electrodes mounted in perspex were used for stimulation of the carotid sinus nerve. In most experiments a rectangular wave electronic stimulator which allowed independent variations of frequency of stimulation (1·5–1000 cyc./sec.), pulse duration (0·02–100 msec.) and voltage output (1–100 V.), was used. In some experiments an ordinary induction coil was used. The carotid sinus nerve was stimulated for periods of 15–35 sec.

In other experiments, the carotid sinus was isolated from the general circulation by ligaturing all branches of the common carotid artery and finally tying off the common carotid itself. A glass cannula was introduced into the common carotid artery and connected to a pressure bottle containing oxygenated buffered Ringer-Locke solution, which allowed rapid changes in pressure to be effected (Moissejeff, 1926; Koch, 1931; Schweitzer, 1937). The pressure in the sinus was recorded from a manometer connected to a side-arm of the cannula.

An alternative method consisted in the perfusion of the isolated carotid sinus by gravity flow. The perfusion fluid left the sinus through a cannula inserted into the external carotid artery. The outflow tube was fitted with a screw clip which allowed variable resistance to flow. The perfusion pressure was recorded from a manometer attached to a side-arm of the outflow cannula.

Blood pressure was recorded from a femoral artery. Heart-rate changes were observed from blood-pressure records obtained with fast kymograph speeds. As an anticoagulant a continuous intravenous drip of heparin was given in many experiments.

A tracheal cannula was inserted. Respiration was recorded by stethograph or closed circuit Krogh spirometer filled with O_2, CO_2 being absorbed by soda lime.

RESULTS

Electrical stimulation of the carotid sinus nerve

Chloralose anaesthesia. In all but two of the experiments, stimulation of the carotid sinus nerve in cats under chloralose anaesthesia, using the rectangular wave stimulator (100 cyc./sec., 0·05–0·5 msec. pulse duration, 1–3 V.) or an induction coil discharge, produced a marked rise in arterial blood pressure (Fig. 1). This rise was rapid and its extent was found to be dependent mainly upon the pulse duration of the stimulus employed. Maximal responses (60–100 mm. Hg) were usually obtained with stimuli of 0·1–0·5 msec. duration.

It was found that the extent of the blood-pressure rise following stimulation of the carotid sinus nerve was independent of the initial blood-pressure level.

The rise of arterial blood pressure thus produced was fairly well sustained during a 30 sec. period of stimulation. On discontinuing the stimulation the blood pressure rapidly fell to its orginal level.

Pressor responses were also obtained with the use of the induction coil stimulator.

In the experiments with the rectangular wave stimulator, the pressor response appeared to be independent of the frequency of stimuli above a threshold value of approximately 50 cyc./sec. Similarly, voltage outputs greater than 1 V. did not appreciably alter the characteristics of the response.

This phenomenon was independent of respiratory variations concomitantly produced by sinus-nerve stimulation. The rate and amplitude of respiration, though commonly increased, varied considerably in different animals. The pressor response to carotid sinus-nerve stimulation, however, was equally well obtained when little or no hyperpnoea was observed (e.g. Fig. 2).

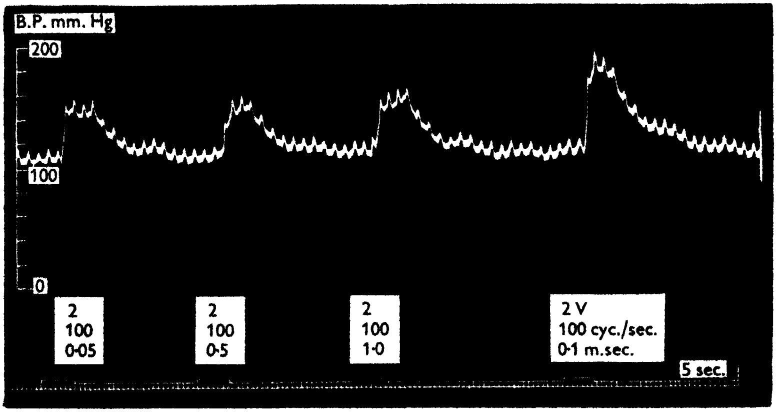

Fig. 1. Cat, chloralose. Records from above downwards: blood pressure, signal, time in 5 sec. Rectangular wave stimulation of right carotid sinus nerve with 100 cyc./sec., 2 V., and pulse durations of 0·05, 0·5, 1·0 and 0·1 msec.

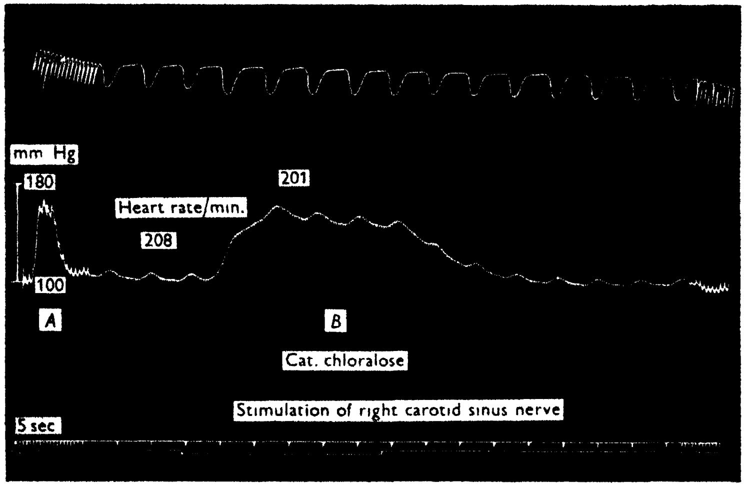

Fig. 2. Cat, chloralose. Records from above downwards: respiration (Krogh spirometer), blood pressure, time in 5 sec., signal. Stimulation of right sinus nerve with induction coil discharge (coil distance 12 cm.) *A*, slow speeds; *B*, fast speeds. Note absence of conspicuous cardiac slowing or respiratory disturbance.

The pressor response was unaffected by double vagotomy, destruction of the contralateral carotid sinus nerve, section of the homolateral glossopharyngeal nerve peripheral to its junction with the carotid sinus nerve, homolateral cervical sympathectomy, sectioning of the homolateral carotid sinus nerve near its origin from the carotid sinus, and bilateral adrenalectomy.

The pressor response to carotid sinus-nerve stimulation was abolished by high cervical spinal transection or section of the glossopharyngeal nerve central to its junction with the carotid sinus nerve.

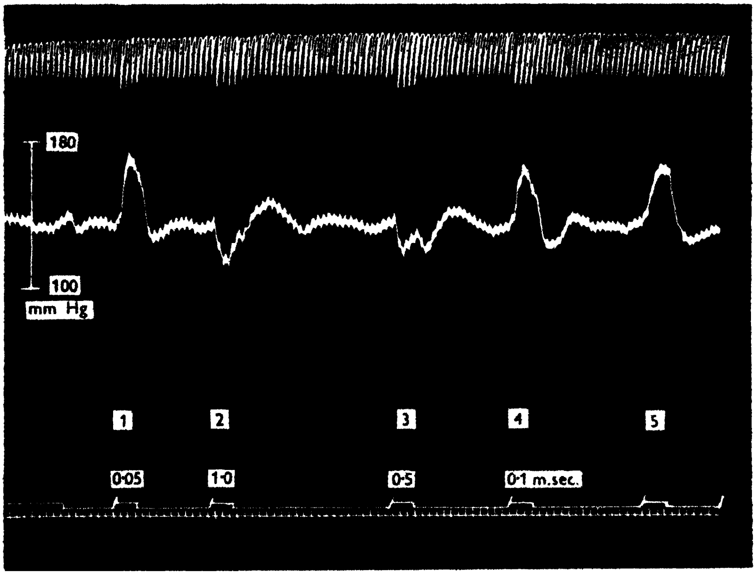

Fig. 3. Cat, chloralose. Records from above downwards: respiration, blood pressure, signal, time in 5 sec. Rectangular wave stimulation of right carotid sinus nerve (100 cyc./sec., 2 V., and pulse durations of 0·05, 1·0, 0·5 and 0·1 msec. Note pressor responses to short, depressor responses to long pulses. Marked response to occlusion of right common carotid artery (signal 5).

Noteworthy changes in heart rate were not observed in experiments where carotid sinus nerve stimulation produced a marked rise in arterial blood pressure (Fig. 2).

The response to carotid sinus-nerve stimulation using the rectangular wave stimulator was found to depend on the pulse duration. Fig. 3 shows the effect of varying the pulse duration of the stimulus, frequency and voltage of stimuli remaining constant (100 cyc./sec., 2 V.). In this experiment pulse durations of 0·05–0·1 msec. produced the usual rise, longer pulses (0·5–1·0 msec.) a fall in arterial blood pressure. In other animals, use of the longer pulse durations (0·5–1·0 msec.), though not producing a fall of blood pressure, failed to cause such a pronounced rise of pressure as was obtained with stimuli of 0·1 msec. duration.

These unexpected results led to an investigation as to the effect of the anaesthetic used.

Decerebrate cats. The experiments were repeated in decerebrate cats, stimulation of the carotid sinus nerve intermittently being carried out over a period of several hours. Invariably, such stimulation caused a marked fall in arterial blood pressure, attended by cardiac slowing. This fall in blood pressure was independent of the frequency or pulse duration of the stimuli used. However, the intravenous injection of ≥0·05 g. of chloralose into these decerebrate cats converted the depressor response to stimulation of the carotid sinus nerve into the definite pressor one described above; as previously, no conspicuous changes in heart rate occurred (Fig. 4).

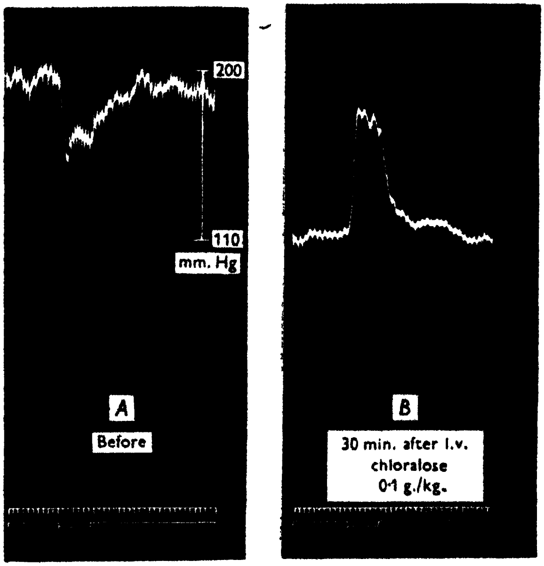

Fig. 4. Decerebrate cat. Records from above downwards: blood pressure, time in 5 sec., signal. Tracing *A*: induction coil stimulation of right carotid sinus nerve (coil distance 15 cm.). Tracing *B*: repeat stimulation 30 min. after intravenous injection of chloralose (0·1 g./kg. body wt.)

Nembutal anaesthesia. Intraperitoneal injection of nembutal, however, in decerebrate cats did not reverse the depressor response to carotid sinus-nerve stimulation (Fig. 5).

In cats, initially anaesthetized with nembutal by intraperitoneal injection, stimulation of the carotid sinus nerve caused a fall in arterial blood pressure, irrespective of frequency or pulse duration of the stimuli used. Similar results were obtained using an induction coil discharge. Subsequent intravenous injection of 0·05–0·2 g. of chloralose converted the depressor response to carotid sinus-nerve stimulation into a marked rise within approximately 20 min. (Fig. 6). During a period of 10–20 min. after chloralose injection, responses to stimulation were indeterminate.

Chloral hydrate. In animals anaesthetized by intravenous injection of chloral hydrate, stimulation of the carotid sinus nerve always caused a fall in blood pressure, often accompanied by cardiac slowing.

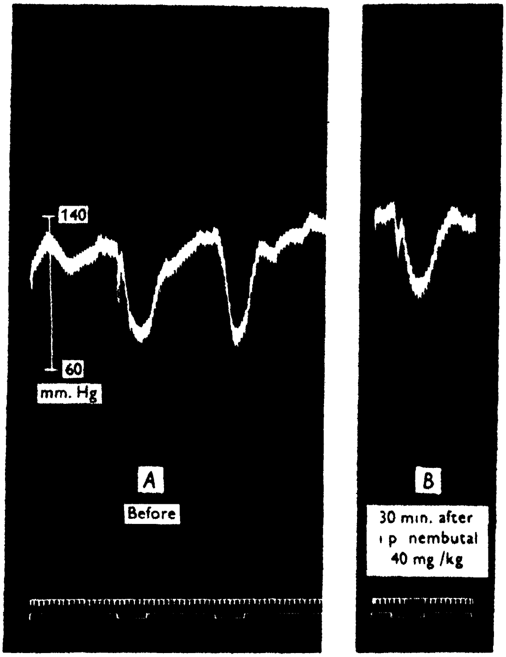

Fig. 5. Decerebrate cat. Records as in Fig. 4. Tracing *A* : induction coil stimulation of right carotid sinus nerve (coil distance 15 cm.). Tracing *B*: repeat stimulation 30 min. after intraperitoneal injection of nembutal (40 mg./kg. body wt.).

From these experiments it would appear that: (1) The rise of arterial blood pressure produced by stimulation of the carotid sinus nerve with pulses of short duration (0·05–0·1 msec.) or with induction coil discharges in cats under chloralose anaesthesia, is a vasomotor centre response. (2) Pulses of longer duration (0·5–1·0 msec.) in chloralosed cats may produce a fall in arterial pressure. (3) The qualitative nature of the response to carotid sinus-nerve stimulation is closely related to the choice of anaesthetic. In the unanaesthetized decerebrate animal, sinus-nerve stimulation causes a fall of arterial blood pressure. The injection of nembutal does not modify this response. Administration of chloralose, however, reverses the fall of arterial blood pressure consequent upon sinus-nerve stimulation into a marked pressor response.

The carotid sinus nerve is a mixed afferent nerve containing afferents from the baroceptors of the carotid sinus and from the chemoceptors of the carotid body. Comroe & Schmidt (1940) have shown that excitation of the chemoceptors of the carotid body by chemical means is attended by slight pressor effects, tachycardia and hyperpnoea.

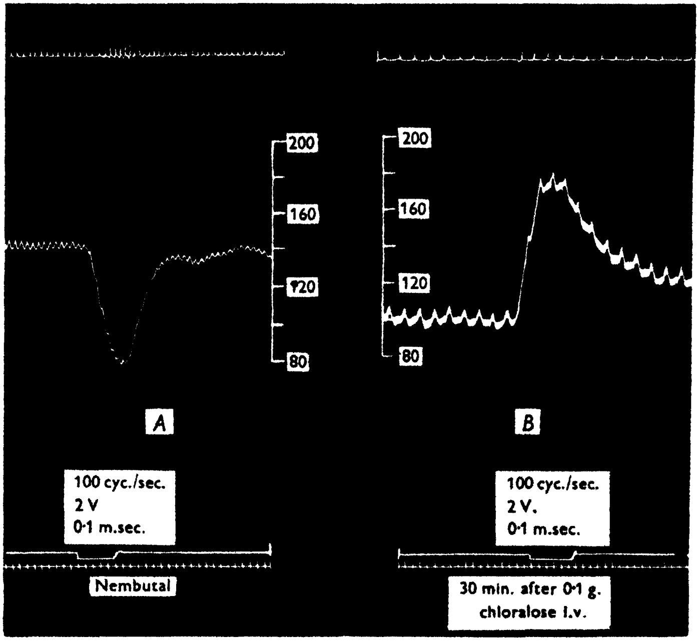

Fig. 6. Cat, nembutal. Records from above downwards: respiration, blood pressure, signal, time in 5 sec. Tracing *A*: rectangular wave stimulation of right carotid sinus nerve (100 cyc./sec., 2 V., 0·1 msec.) before, tracing *B*: 30 min. after intravenous injection of 0·1 g. chloralose.

Electrical stimulation of the carotid sinus-nerve trunk might be expected to cause excitation of both types of afferent fibre. There are various possible ways in which chloralose anaesthesia might produce the decisive modification of the response to electrical stimulation: (1) By sensitization of baroceptors which reflexly raise the blood pressure. Such baroceptors, functionally inactive under normal conditions, might become prominent in conditions of arterial hypotension. Though the existence of such pressor afferents has been denied (Koch, 1931; Schweitzer, 1937) it was thought necessary to reinvestigate this point. (2) By modifying the response of the vasomotor centre to carotid sinus-nerve stimulation by suppressing the inhibitory effects of baroceptor excitation on the centre. Chemoceptor stimulation would thus be revealed and then produce a rise in arterial blood pressure. (3) By diminishing the excitability of the afferent fibres arising from the baroceptors. (4) By affecting in some way the efferent part of the reflex arc involved in the vasomotor response to carotid sinus-nerve stimulation.

Some of these theoretical possibilities have been experimentally investigated.

Effect of altering pressure in carotid sinus

Absence of pressor fibres in the carotid sinus nerve. This problem was investigated by subjecting one isolated carotid sinus preparation to changes of static internal pressure. Cats under chloralose anaesthesia were used. Both vagi were cut and the carotid sinus nerve of the opposite side was sectioned. Koch (1931) and Schweitzer (1937) showed that a threshold intrasinusal pressure of 70–80 mm. Hg is required to produce excitation of the baroceptors in the dog. It was therefore decided to maintain an initial pressure of 70 mm. Hg in the isolated carotid sinus in these experiments.

Sudden lowering of the intrasinusal pressure from 70 to 0 mm. Hg did not produce any change in systemic blood pressure. It was therefore concluded that the carotid sinus nerve does not contain true pressor fibres arising from baroceptors. The rise of the arterial blood pressure produced by certain types of electrical stimulation of the sinus nerve trunk must have been due to some other mechanism.

Modification of vasomotor centre response by chloralose. Nembutalized cats, bilaterally vagotomized, were used in these experiments. The contralateral sinus nerve was cut and the remaining sinus isolated as previously. Raising the intrasinusal pressure from 70 to 220 mm. Hg produced a marked fall in arterial pressure. This response was still obtained over 2 hr. after commencing the experiment (Fig. 7). In the experiment illustrated by Fig. 7, 0·06 g. of chloralose was then injected intravenously. Five minutes later, raising the intrasinusal pressure from 70 to 220 mm. Hg only produced a slight fall in arterial pressure; 20 min. later the response to an increase of pressure in the carotid sinus was completely abolished (Fig. 7). On electrical stimulation of the corresponding carotid sinus nerve, marked pressor responses were obtained.

In this type of experiment chloralose did not have access to the baroceptors or to the afferent fibres of the carotid sinus nerve, which mainly receive their blood supply from capillaries of the ascending pharyngeal system. Consequently, the effect of chloralose must have been exerted on structures other than the afferent side of the reflex arc. A possible criticism of this conclusion would be that the abolition of the response to a rise of intrasinusal pressure was merely due to death of the baroceptors. However, this experiment was repeated several times with similar results. Complete abolition of the response to static pressure changes in an isolated sinus preparation after intravenous injection of chloralose was not invariable, but partial abolition always occurred.

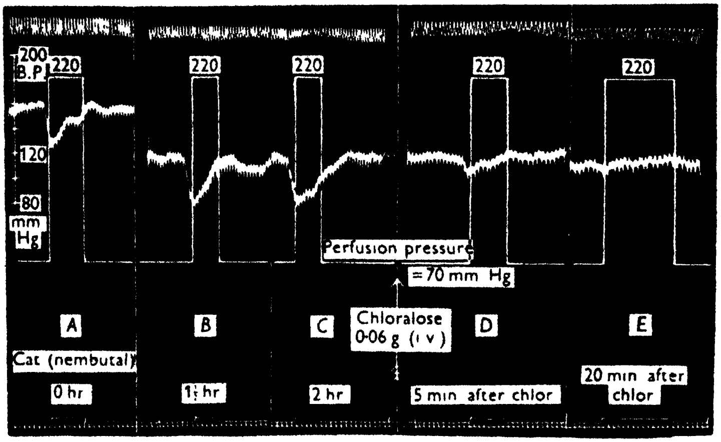

Fig. 7. Cat, nembutal. Isolated right sinus preparation. Other buffer nerves cut. Records from above downwards: respiration, blood pressure, hydrostatic pressure in right sinus, signal, time in 5 sec. At *A*, *B* and *C* responses to increase in intrasinusal pressure from 70 to 220 mm. Hg at 0, ½ and 2 hr. At arrow, intravenous injection of 0·06 g. chloralose.. Note greatly diminished response to sudden rise of intrasinusal pressure after 5 min. (*D*); response completely abolished after 20 min.

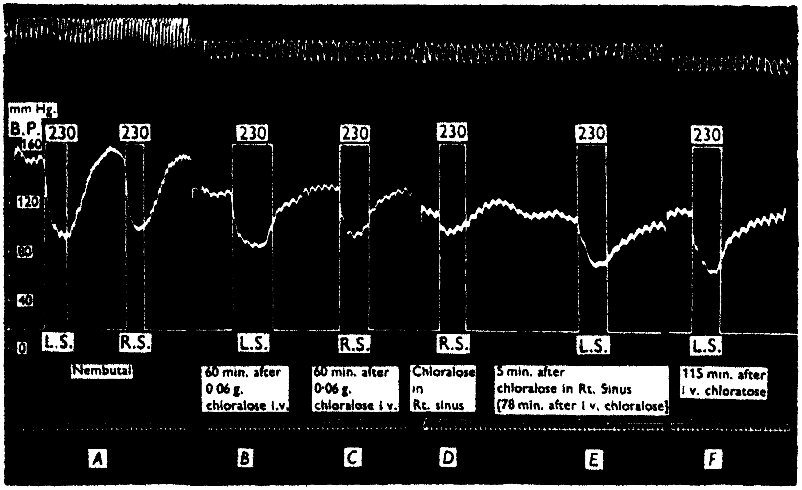

Fig. 8. Cat, nembutal. Both vagi cut. Isolated left and right carotid sinus preparations. Records as in previous figure. *A*: responses to left and right intrasinusal pressure rises from 0 to 230 mm. Hg. *B* and *C*: responses to intrasinusal pressure rises, 60 min. after intravenous injection of 0·06 g. chloralose. Between *C* and *D* inject chloralose into right sinus preparation. *D*: response to pressure increase in right sinus diminished. *E* and *F*: responses to pressure increases in left sinus unaffected.

These results presumably explain our failure on many occasions to obtain responses to rises of intrasinusal pressure in chloralosed cats. Invariably, in such animals, stimulation of the carotid sinus nerve trunk produced a pressor response.

Thus it would appear that chloralose affects the response of the vasomotor centre or the efferent part of the vasomotor reflex arc. Obviously, these experiments do not exclude the possibility that chloralose affects the baroceptors in the intact animal. This possibility was next investigated.

The effect of chloralose on the response of the baroceptors. Bilateral carotid sinus gravity perfusion preparations were used in these experiments. Cats under nembutal anaesthesia were bilaterally vagotomized. Both carotid sinus preparations were alternately subjected to rises in internal pressure. Typical depressor responses were recorded. Chloralose was then injected intravenously, and it was found that the blood-pressure responses to alternate increases of pressure in the perfused sinuses were almost identically diminished (Fig. 8). Chloralose in an amount sufficient to give a final concentration of 0·03 % was then added to the solution perfusing one carotid sinus. Within 5 min. exposure of that sinus preparation to chloralose the response to an increase in pressure was further reduced, but the response from the other sinus was unchanged.

Such an experiment shows that, in addition to a modification of the vasomotor centre response to afferent nerve stimulation seen in cats under chloralose anaesthesia, there is the further possibility of a direct action of the chloralose on the baroceptors themselves.

Clearly, the local injection of chloralose into the isolated and perfused carotid sinus can exert no effect on the efferent side of the vasomotor reflex arc.

In two chloralosed animals normal depressor responses to rise in intrasinusal pressure were obtained; electrical stimulation of the carotid sinus nerve in these animals caused only a fall in blood pressure. These results were obtained only in animals with perfused carotid-sinus preparations in which there exists a possibility of a gradual decrease of the chloralose concentration in the baroceptor tissue under conditions of continuous perfusion with Ringer-Locke solution.

DISCUSSION

It is well known that a rise of pressure in the carotid sinus stimulates the baroceptors of the sinus causing a reflex fall in arterial blood pressure. Von Euler, Liljestrand & Zotterman (1941) have shown that such reflex effects may be obtained in the cat when the perfusion pressure is raised in the carotid sinus. It was therefore surprising to find that, in the cat under chloralose anaesthesia, electrical stimulation of the carotid sinus nerve either by means of an induction coil discharge or a rectangular wave electronic stimulating device, can produce dramatic pressor responses.

In some of these cats, by varying only the pulse duration of the individual stimuli, we were able to elicit either pressor or depressor effects on the arterial blood pressure. This differentiation of the effects of carotid sinus-nerve stimulation may be related to the different excitability time constants of the fibres composing the nerve trunk. Zotterman (1935) has presented evidence of the presence of small and large myelinated fibres in the carotid sinus nerve of cats, assuming that the larger fibres are derived from the baroceptors and the smaller components from the chemoceptors. A detailed histological examination of the composition of the carotid sinus nerve in various species is at present in progress.

Analysis of the pressor response obtained in cats under chloralose anaesthesia has shown it to be an effect involving activity of the vasomotor centre.

The experiments on the isolated carotid sinus preparations prove conclusively that the pressor response to sinus nerve stimulation was not due to stimulation of true pressor fibres from the baroceptors.

The explanation of the pressor effect of electrical stimulation of the carotid sinus nerve in chloralosed cats, therefore, involved a consideration of a possible abolition, partial or complete, of the classical inhibitory influence of the carotid sinus mechanism on vasomotor centre activity, and/or a sensitization of chemoceptor effects on the vasomotor centre. It is shown in our experiments on the effects of perfusion of solutions containing chloralose through the isolated carotid sinus that the site of action of the anaesthetic could not have been the peripheral efferent side of the reflex arc involved in these vasomotor phenomena.

Evidence was presented indicating that chloralose inhibits the effects of baroceptor stimulation on the vasomotor centre. In animals with both carotid sinuses isolated from the circulation, responses to alternative intrasinusal pressure rises were invariably diminished following intravenous injection of chloralose, as compared with the results, previously obtained in the same animal under initial nembutal anaesthesia.

It was further shown, by perfusion of Ringer-Locke containing chloralose through one isolated carotid sinus, that the response of that sinus preparation to an augmentation of perfusion pressure was markedly diminished, thus showing that chloralose also decreased the sensitivity of the baroceptors to pressure stimulation.

It is therefore concluded that intravenous injection of chloralose in intact cats both diminishes the sensitivity of the baroceptors to hydrostatic pressure changes and inhibits the vasomotor centre response to baroceptor stimulation. Under such conditions, carotid sinus-nerve trunk stimulation essentially excites only the afferents arising from the chemoceptors of the carotid body, causing a rise of blood pressure.

On several occasions, section of the carotid sinus-nerves in cats under chloralose anaesthesia, both vagi being already cut, has caused a fall of arterial blood pressure. Such results indicate the preponderance of tonic chemoceptor afferent discharge upon the vasomotor centre.

If this interpretation of our experimental results be correct, it is incumbent upon us to explain the fact that occlusion of both common carotid arteries in cats under chloralose anaesthesia always causes a marked rise in arterial pressure. Much of this effect is, in our belief, due to the stimulation of the chemoceptors of the carotid body by the changes in the composition of the stagnant blood contained therein.

Marked depressor responses to rises in intrasinusal pressure can occasionally be obtained in cats under chloralose anaesthesia, but such effects were only recorded when electrical stimulation of the carotid sinus nerve trunk produced a fall in blood pressure, especially when stimuli of long duration were used. Von Euler, Liljestrand & Zotterman (1941) have also obtained depressor responses to rises in intrasinusal pressure, using cats under chloralose anaesthesia.

Recent work has shown that the results of these experiments on cats cannot be obtained in the case of the dog or the rabbit.

SUMMARY

1. Stimulation of the carotid sinus nerve trunk in cats under chloralose anaesthesia by means of induction coil discharges or a rectangular wave electronic stimulator produced marked rises in arterial blood pressure with pulses of short duration (0·05–0·1 msec.), and hyperpnoea, but only insignificant changes in heart rate. Pulses of longer duration (0·5–1·0 msec.) occasionally caused a fall in blood pressure. These effects involved the activity of the vasomotor centre.

2. Stimulation of the carotid sinus nerve in unanaesthetized decerebrate or in nembutalized cats, or in cats under chloral hydrate anaesthesia, invariably lowered the blood pressure, irrespective of the duration of the stimuli used. Subsequent intravenous injection of chloralose in such animals converted the previous depressor response to stimulation into a pressor response.

3. The carotid sinus nerve does not contain pressor fibres.

4. Responses to rises of intrasinusal pressure in nembutalized cats were diminished by intravenous injection of chloralose. In addition, perfusion of the isolated carotid sinus with Ringer-Locke solution containing chloralose diminished the response to increase in hydrostatic pressure.

5. Chloralose diminishes in cats the sensitivity of the vasomotor centre and of the baroceptors of the carotid sinus to changes of intrasinusal pressure.

6. The pressor response to electrical stimulation of the carotid sinus nerve trunk in cats under chloralose anaesthesia is due to excitation of the chemoceptor afferents contained in the nerve.

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THE ROLE OF CARBON DIOXIDE IN OXYGEN POISONING

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Oxygen convulsions are believed by many workers to be caused by an accumulation of carbon dioxide in the body, one suggestion being that the oxyhaemoglobin is not reduced in the capillaries in contact with the tissues, and this interferes with the mechanism for the transport of carbon dioxide. In addition, there is some evidence that the toxic properties of oxygen are increased as the tension of carbon dioxide breathed at the same time is increased. Campbell (1929) showed that animals breathing oxygen showed a rise in carbon dioxide tissue tensions but made no systematic study, particularly with regard to rate of rise and value reached in convulsions. Taylor (1949), studying the effect on cats of breathing oxygen at atmospheric pressure, obtained evidence to support the view that carbon dioxide plays some part in the toxic action of oxygen.

The object of the following experiments was to seek further information on the effect of oxygen under pressure on the behaviour of carbon dioxide in the tissues.

METHODS

Oxygen and carbon dioxide tensions in the tissues were measured by the method of injection, developed by Campbell (1924), of 40–50 ml. of nitrogen subcutaneously. The gas gradually reached equilibrium with the tissue gases. Depots of gas were thus established, samples withdrawn when required for analysis by the method described by Campbell & Taylor (1935), and the oxygen and carbon dioxide tensions calculated. If the animal was under increased pressure it was decompressed to atmospheric pressure before a sample was taken. Cats were used and the nitrogen was injected subcutaneously in the back. In this site the gas does not spread as it does if it is injected subcutaneously in the region of the abdomen. 20–30 ml. of nitrogen were injected and the gas left for at least 5 hr. in order that equilibrium with the tissue gases should be established. The animal was then placed in a small cylindrical pressure chamber of capacity 138 l. and the oxygen pressure raised to the required value. Control experiments were carried out by subjecting animals to equivalent pressures in air. In order that the nitrogen in the injected bubble should not be rapidly removed the nitrogen in the chamber was not washed out of the chamber by oxygen so that the partial pressure of nitrogen remained constant at about 610 mm. Hg. As soon as a convulsion occurred the gas in the chamber was rapidly decompressed to atmospheric pressure, the animal removed, and a sample of the subcutaneous gas taken for analysis. Values were thus obtained for the tissue carbon dioxide and oxygen tensions when a convulsion occurred.

Next the rate of rise of oxygen and carbon dioxide tensions in the tissues under these conditions was determined. Two methods were employed.

(*a*) A series of determinations of oxygen and carbon dioxide tissue tensions after varying times of exposure to high oxygen pressure were made. A bubble of nitrogen was injected into the animal and when equilibrium with the tissue gases was established the animal was placed in oxygen at the required pressure for 0·5 hr. It was then decompressed and a sample of gas removed for analysis. The animal was left for 24 hr. for tissue gas tensions to return to normal and the experiment repeated. The time of exposure to oxygen under pressure was, however, lengthened to 1 hr. before decompressing and taking the gas sample. This was repeated for gradually increasing times of exposure until a convulsion intervened.

(*b*) The results obtained by (*a*) may be open to objection on the grounds that day to day variations in the state of the animal and of the ambient conditions may produce variations in the rate at which equilibrium is achieved. The experiment also took considerable time and the following procedure was therefore adopted. An animal, with a bubble of gas in equilibrium with the tissues was placed in the pressure chamber in oxygen at the required pressure for 0·5 hr. It was then rapidly decompressed, a sample of gas withdrawn for analysis and the oxygen pressure raised to its original value as soon as the animal was back in the chamber (the whole operation occupied less than 1·5 min.). At the end of another 0·5 hr. the animal was again decompressed and another gas sample taken for analysis. This procedure was carried out until the animal convulsed. The value found at the end of the second 0·5 hr. exposure was taken as being due to a total compression time of 1 hr. and so on. The whole experiment was repeated using different oxygen pressures.

In order to test the effect of various factors, such as intermittent rapid decompression and recompression, added carbon monoxide, and added carbon dioxide to the inspired oxygen, on the oxygen convulsion time of an animal, it was necessary to 'calibrate' the animal for convulsion time in oxygen alone. This was done by exposing the animal on alternate days for about 2–3 weeks to a certain oxygen pressure and noting the time of exposure required to produce an oxygen convulsion. The effect of the various factors on convulsion time can then be estimated.

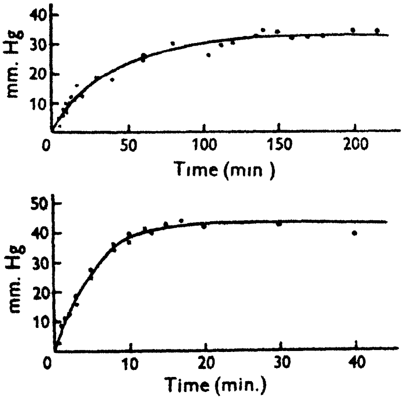

Fig. 1. Changes in oxygen and carbon dioxide tensions in the tissues after the injection of nitrogen. *Above*, oxygen; *below*, carbon dioxide.

RESULTS

Equilibrium rate of subcutaneous gas bubbles. Fig. 1 shows the rate at which equilibrium between the nitrogen bubble and the tissue gases is reached. This equilibrium is established in about 18–20 min. for carbon dioxide and about

2·5 hr. for oxygen. The normal values for cats, breathing air, in the subcutaneous region used, were found to be 28–32 mm. Hg for oxygen and 40–43 mm. Hg for carbon dioxide.

Oxygen and carbon dioxide tissue tensions after an oxygen convulsion. The experiment was carried out as described previously and the results are given in Table 1. The results agree with the observations made by Campbell (1929) that exposure of rabbits to high oxygen tensions results in increased tissue carbon dioxide tensions. The values given are calculated on the assumption that during decompression little or no change occurs in the oxygen or carbon dioxide concentration in the bubble. The time of decompression is so short (30 sec.) that little change could be expected in that time (see Fig. 1). It should be pointed out that the figures for oxygen and carbon dioxide tissues shown in Table 1 are lower than the real tensions in the tissues since under these conditions equilibrium is not established. The results, however, give a good indication that the rise in these tissue tensions is a significant one.

TABLE 1. Values of carbon dioxide and oxygen tissue tensions after convulsion

			Oxygen		Carbon dioxide	
Cat no.	Pressure of oxygen (lb./sq.in.)	Convulsion time hr. min.	Percentage*	Calculated tension (mm. Hg)	Percentage	Calculated tension (mm. Hg)
1	55†	3 14	12·3	410·0	7·2	246·0
2	55	3 05	11·8	393·0	6·4	213·0
3	55	2 45	9·03	301·0	5·5	183·0
4	55	2 55	10·3	343·0	6·0	200·0
5	55	3 45	12·3	410·0	6·8	226·0
6	55	2 45	12·9	430·0	6·1	203·3

* The normal value for an animal breathing air is just under 5% for oxygen and just over 5% for carbon dioxide.

† The value of 55 lb. abs. was chosen because at this pressure the convulsion time is comparatively long. This is necessary as the time taken for the oxygen tension in the bubble to reach equilibrium with the tissues is a slow process, and with short convulsion times the value obtained will not even approximate to the true value. It is probable that the value given for oxygen tension in Table 1 will, for this reason, be always on the low side.

The results of control experiments on cats taken to equivalent pressures in air are shown in Table 2. They show that although a small rise in carbon dioxide and oxygen tensions was found when air is used instead of oxygen at high pressure, these rises are in no way comparable with the figures obtained in oxygen.

TABLE 2. Effect of breathing air under pressure on oxygen and carbon dioxide tissue tensions

Cat no.	Pressure (lb./sq.in.)	Time of exposure hr. min.	Percentage	Calculated tissue tensions (mm. Hg)	Percentage	Calculated tissue tensions (mm. Hg)
1	67	3 14	1·30	44·0	1·40	47·3
2	67	3 05	1·34	45·5	1·60	54·3
4	67	2 55	1·27	43·1	1·60	54·3
6	67	2 45	1·21	41·1	1·3	44

The rate of rise of carbon dioxide and oxygen gas tensions in the tissues. Experiments were carried out on three cats by methods (*a*) and (*b*) described previously. The results for one animal are shown in Fig. 2. They show the rate of change of oxygen and carbon dioxide tensions in the bubble and the equivalent calculated tissue tensions.

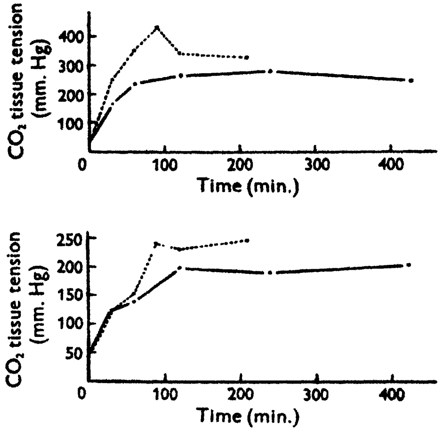

Fig. 2. Rate of rise of tensions of oxygen and carbon dioxide in the tissues during the breathing of oxygen at high pressure. *Above*, oxygen; *below*, carbon dioxide; *broken line*, oxygen 55 lb./sq.in., nitrogen 12 lb./sq.in.; *continuous line*, oxygen 43 lb./sq.in., nitrogen 12 lb./sq.in.

The effect of intermittent rapid decompression on convulsion time. 'Calibrated' cats with a reasonably constant convulsion time were selected for this experiment. These were exposed to oxygen at high pressure, were decompressed and then rapidly recompressed at approximately 30 min. intervals. The time taken from beginning of decompression to end of recompression was approximately 1·5 min. The results are recorded in Table 3. No further test of significance is

Table 3. Effect of intermittent decompression and compression in convulsion time

Cat no.	1	2	7	6
Pressure of oxygen	55 lb./sq.in.	—	—	—
Convulsion time (min.)	225	185	135	165
	195	225	150	170
	180	149	120	165
	210	210	127	120
	225	235	210	135
	—	195	—	—
Mean	207	216·5	153·7	150·9
Standard deviation	19·6	28·4	34·8	19·6
Coefficient of variation (%)	9·5	11·3	22·6	13·0
Convulsion time with decompression (min.)	230	220	150	160
Difference from mean	+25	+3·5	−3·7	+9·1
Difference (%)	+11·1	+1·6	−2·4	+6·0

required for these figures, since in no case does the observed difference from the mean exceed two standard deviations. In fact, in only one case does it exceed one standard deviation. The correctness of the assumption made when using method (*b*) (above) for estimating the rate of rise of tissue oxygen and carbon dioxide tensions is thus supported.

Effect of carbon monoxide on oxygen convulsion time. Whole blood exposed to a partial pressure of oxygen of three atmospheres has 6 ml. % of oxygen in simple solution. This is the normal amount lost per 100 ml. of circulating blood in man and some other animals at rest. In these circumstances the needs of the body for oxygen can therefore be satisfied without any reduction of oxyhaemoglobin. Thus the potential alkalinity of haemoglobin is completely lost from the point of view of carbon dioxide transport. If part of the oxyhaemoglobin is replaced by carboxyhaemoglobin and sufficient oxygen is supplied in solution in the plasma to satisfy the normal oxidation processes in the body it may be that the toxic effect of oxygen in the system would be unaffected. Experiments were carried out with this in mind. Animals whose convulsion times were well known (selected animals) were exposed to an atmosphere of oxygen (55 lb./sq.in.) in which the relative partial pressures of carbon monoxide : oxygen are 1 : 506. Since the relative affinity for haemoglobin of carbon monoxide and oxygen is 310 : 1, then one would expect that under these conditions the relative amounts of carboxyhaemoglobin and oxyhaemoglobin would be approximately 3 : 5. Table 4 gives the results. It will be seen that the time to convulsions is scarcely altered, i.e. the toxicity of this particular pressure of oxygen is not appreciably modified.

TABLE 4. Effect of added carbon monoxide on oxygen convulsion time

Cat no.	Mean convulsion time at 55 lb./sq.in. of oxygen		Convulsion time when breathing CO in concentration of 0·2 % CO at same O_2 pressure	
	hr.	min.	hr.	min.
1	3	30	3	30
5	3		3	15
2	3	20	3	29
3	2	27	2	50

The effect of added carbon dioxide on the oxygen convulsion time of animals. This effect has been studied by many workers and the hastening of convulsions at a given high oxygen pressure has been noted. In particular, Haldane & Spurway (1943) emphasize this fact. These authors carried out experiments on man and they concluded that 'the effect of carbon dioxide in enhancing the toxicity of high pressure oxygen begins at a partial pressure corresponding to about 3 % of one atmosphere'. Table 5 shows the effect of added carbon dioxide upon the time to convulsion of a cat breathing oxygen at pressure. It will be noted that the convulsion time of this cat shortens to 10–13 min. and does not decrease any more, however much carbon dioxide is added. All animals used in this experiment (4) show exactly the same effect, whatever the original convulsion time in pure oxygen. In other words, the animals all had a minimum convulsion time and further addition of carbon dioxide had no effect. Pratt (1944) found an exactly similar effect by increasing the oxygen pressure. He exposed cats to increasing oxygen pressures and noted the convulsion times. These fall to a minimum at about 80–100 lb./sq.in. when further increase of oxygen pressure fails to decrease the time (approx. 10–12 min.).

TABLE 5. Effect of added carbon dioxide on convulsion time of a cat exposed to high oxygen pressure (55 lb./sq.in.)

Partial pressure of added carbon dioxide (% of 1 atmosphere)	Convulsion time (min.)
0	31, 41, 38, 30, 26, 32, 42, 25
1·40	42, 25
2·35	15, 23, 18
3·30	16, 15, 22
4·70	12
7·00	10
10·30	13
12·20	13

Two other animals exposed to 63 lb./sq.in. of oxygen together with carbon dioxide of approximately 11% of an atmosphere show a minimum convulsion time of 3·5–5 min. These cats were exposed to oxygen pressures of 21 lb./sq.in., 29 lb./sq.in. and 36 lb./sq.in. together with 11% of an atmosphere of carbon dioxide and again the animals convulsed in 3·5–5 min. These oxygen pressures alone failed to produce oxygen convulsions, however long (up to 2 hr.) the animal was exposed. This is of interest in that Taylor (1949) has shown that convulsions may be produced in cats breathing oxygen at atmospheric pressure and that carbon dioxide tissue tensions in such animals have a high value. Recent work shows that the subcutaneous injection of carbon dioxide (about 300 c.c.) into cats also has some effect in shortening their convulsion time in oxygen.

DISCUSSION

A full discussion on the etiology of reactions to oxygen at high pressure is to be found in reviews of the subject by Stadie, Riggs & Haugaard (1944) and Bean (1945). Bert (1878) and subsequent workers have shown that the outstanding sign of the effects of exposure to high oxygen pressures is a convulsion associated with respiratory difficulty. Thompson (1889) suggested that an accumulation of carbon dioxide in the body as a result of an interference with normal diffusion processes was responsible for these convulsions. Hill & Macleod (1903) also produced evidence to show that compressed air impeded the diffusion of carbon dioxide in the lungs. Gesell (1923) pointed out that a failure of reduction of oxyhaemoglobin should interfere with the normal function of haemoglobin in the transport of carbon dioxide from the tissues by the blood. This would lead to an accumulation of carbon dioxide in the tissues. As pointed out earlier on in this paper, the amount of oxygen dissolved in the plasma when blood is exposed to oxygen at a pressure of three atmospheres (45 lb./sq.in.) is sufficient for the normal needs of the body; there will then be no reduction of oxyhaemoglobin. The base provided by the reduction of oxyhaemoglobin, and which normally serves for the transport of most of the carbon dioxide from the tissues would, under these conditions, not be available.

The first direct evidence of accumulation of carbon dioxide in the tissue was provided by Campbell (1929). He measured oxygen and carbon dioxide tissue gas tensions by the method described earlier in this paper. Campbell & Hill (1931) suggested that it is the accumulation of carbon dioxide which causes the convulsion, but that this increased carbon dioxide tension may be due to vasoconstrictor effect of oxygen. Hill (1933) showed that a preliminary exposure of an animal to a mixture of 5% carbon dioxide and 95% oxygen lowered the critical pressure at which convulsions occurred. On the other hand, Shaw, Behnke & Messer (1934) reported that anaesthetized animals when exposed to high oxygen pressure and caused to over-ventilate artificially still gave signs of oxygen poisoning. This over-breathing should lead to a fall in alveolar carbon dioxide and thus to a fall in tissue carbon dioxide tension. Behnke & Shaw (1937) therefore concluded that carbon dioxide had little effect on the production of oxygen poisoning. The assumption that tissue carbon dioxide and alveolar carbon dioxide are always interdependent would obviously not be valid if a breakdown in carbon dioxide transport from the tissues took place. In fact, under these latter conditions one would expect a lowered alveolar carbon dioxide content. Bohr & Bean (1942) subjected tracheotomized, urethanized or decerebrate cats to controlled artificial respiration in oxygen at pressures of six atmospheres. They found that over-breathing postponed the onset of reactions to high pressure oxygen. This may be explained on the basis of an increased carbon dioxide diffusion gradient from the plasma to the alveolar gas. On the other hand, this over-ventilation would hasten the uptake of oxygen. If the rate of attainment of a certain oxygen partial pressure in the tissues controlled the convulsion time one would expect this over-ventilation to hasten the onset of a convulsion. Further evidence of the part carbon dioxide plays in oxygen poisoning is provided by the work of Bean (1929, 1931) who found a definite decrease in pulse rate in dogs subjected to 4–5 atmospheres of oxygen which he compared to a similar result from the administration of carbon dioxide.

The present work produces confirmatory evidence of the rise in tissue carbon dioxide tensions produced by exposure to oxygen at high pressure. This carbon dioxide tension reaches its maximum some time before a convulsion occurs and this would suggest that if a rise of carbon dioxide is a causative factor in producing a convulsion the critical factor is not a particular value of the carbon dioxide tension but rather of this value and the time the tissue tension is raised. However, it may be that in other tissues, notably the brain, the rate of rise of carbon dioxide tissue tension is different. Experiments on the tissue gas tensions in the brain under similar conditions of high oxygen pressure are being undertaken. It will also be noted, in the experiments described, that exposure to air at equivalent pressures, raises the oxygen and carbon dioxide tissue tensions to a small extent. These values, however, are not comparable with those obtained by breathing oxygen at the same pressure. The small rise in carbon dioxide tissue tension may be due to interference with the diffusion of carbon dioxide in the alveoli owing to the increased density of the inspired gases. This effect would also come into play when oxygen at high pressure is breathed but, as suggested by Gesell (1923), is probably insignificant compared with the interference with carbon dioxide transport.

No effect on convulsion time was found when cats breathed a mixture of carbon monoxide and oxygen under pressure so that the calculated carboxyhaemoglobin: oxyhaemoglobin ratio was 3 : 5. These convulsions had the appearance of normal oxygen convulsions. It thus appears that oxygen transported by oxyhaemoglobin has little effect on the production of oxygen convulsions, but on the other hand from the point of view of carbon dioxide transport it would be of little moment whether the haemoglobin were present as oxy- or carboxyhaemoglobin. Since carrying out this work it has come to my notice that Lorrain Smith (1899) has already considered this point. He tried to show that the toxic effect of oxygen was related to its tension in the inspired air, and not to its quantity in the blood. By the latter expression he presumably meant the relative quantity of oxyhaemoglobin, and he does not take into account the oxygen dissolved in the plasma. He found that larks developed convulsions as usual when exposed in a chamber containing 0·4 % carbon monoxide as well as three atmospheres of oxygen, although, as he says, 'the arterial blood at the end of the exposure was only 38 % saturated with oxygen'.

SUMMARY

1. The work of Campbell (1929), who showed that when animals are exposed to high oxygen partial pressure then both oxygen and carbon dioxide tissue tensions reach high values, is confirmed.

2. Intermittent rapid decompression and compression have little effect on the rate of rise of these gaseous tensions in the tissues, and have no effect on the resultant time of exposure required to produce convulsions (in cats) compared with that of continuous exposure.

3. Breathing a concentration of 0·2 % carbon monoxide such that, by calculation, the relative amounts of carboxyhaemoglobin and oxyhaemoglobin were 3 : 5 (approx.) did not modify the time to convulsions of animals exposed to high oxygen partial pressures.

4. The well-known effect of carbon dioxide on the time required to produce oxygen convulsions is further discussed. Carbon dioxide given by injection was also found to have some effect. Much lower carbon dioxide tensions than those usually considered to be effective are shown to produce a shortening of the time required for the onset of oxygen convulsions. This effect is most marked in cats whose convulsion time in oxygen, without added carbon dioxide, is comparatively long. The convulsion time was found to reach a limiting value however much carbon dioxide was added.

I wish to express my gratitude to Dr C. L. G. Pratt for all the help and advice he has given during the course of this work.

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BLOOD-PRESSURE RESPONSES TO ELECTRICAL STIMULATION OF THE CAROTID SINUS NERVE IN DOGS AND RABBITS

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The present authors have recently reported the effects of electrical stimulation of the carotid sinus nerve in cats (Neil, Redwood & Schweitzer, 1948, 1949). Stimulation of the carotid sinus nerve in these animals was found to produce blood-pressure responses which were dependent on the anaesthetic and on the type of stimulus employed. Thus, under chloralose anaesthesia, rectangular wave stimulation of the nerve with pulses of short duration (0·05–0·1 msec.) resulted in marked rises in arterial blood pressure. Pulses of longer duration (0·5–1·0 msec.) usually produced a rise in blood pressure but occasionally depressor responses were observed. Under nembutal (pentobarbital sodium) anaesthesia, however, or in decerebrate cats stimulation of the sinus nerve invariably lowered the blood pressure irrespective of the pulse duration of the stimulus employed.

Analysis of these phenomena suggested that chloralose anaesthesia diminishes the sensitivity of the vasomotor centre to baroceptor stimulation. Some evidence was also found that the sensitivity of the baroceptors of the carotid sinus to changes of intrasinusal pressure was diminished by chloralose. It was concluded that the pressor responses to electrical stimulation of the carotid sinus nerve trunk in cats under chloralose anaesthesia was due to excitation of the chemoceptor component of the nerve.

This study has been extended in an attempt to ascertain whether chloralose anaesthesia causes a similar modification of the response to carotid sinus nerve stimulation in rabbits and in dogs. In view of the position of chloralose as the anaesthetic of choice in the study of vasomotor phenomena it is of importance to exclude effects directly attributable to the anaesthetic used.

METHODS

Fourteen rabbits and nine dogs were used in these experiments. The animals were anaesthetized with chloralose (0·08–0·1 g./kg. body weight intravenously) or nembutal (Pentobarbitone) (35–40 g./kg. body weight, intraperitoneally).

The carotid sinus nerve was prepared for stimulation as previously described. Fine shielded silver wire electrodes mounted in perspex were used for stimulation of the nerve. In most experiments a rectangular wave electronic stimulator was used which allowed independent variations of frequency of stimulation (1·5–1000 cyc./sec.), pulse duration (0·02–100 msec.), and voltage output (1–100 V.). In some experiments an ordinary induction coil was used. The carotid sinus nerve was stimulated for periods of 20–30 sec.

Blood pressure was recorded from the femoral artery. Heparin was used in some experiments as an anticoagulant.

RESULTS

Electrical stimulation of the carotid sinus nerve in rabbits. In all experiments, stimulation of the carotid sinus nerve in rabbits under chloralose anaesthesia, using the rectangular wave stimulator (100 cyc./sec., 0·05–1·0 msec., 2 V.) produced a marked fall in arterial blood pressure. Induction coil stimulation of the nerve yielded similar results. Bilateral vagotomy and contralateral sinus nerve section did not affect the direction of the blood-pressure response to sinus nerve stimulation.

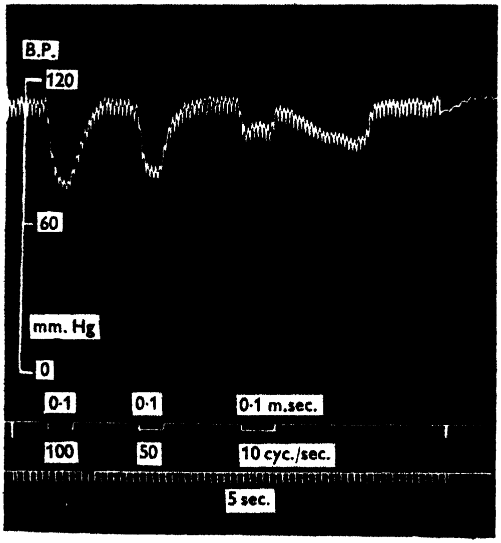

Fig. 1. Rabbit under chloralose anaesthesia. Records from above downwards: blood pressure, signal, time in 5 sec. left sinus denervated, left vagus cut. Stimulation of right carotid sinus nerve with 100, 50 and 10 cyc./sec., 0·1 msec. pulse duration and 2 V. Note absence of pressor responses.

In a number of experiments the frequency of stimulation was altered, voltage and pulse duration being maintained constant (2 V., 0·1 msec.). The threshold frequency of stimulation was found to be 10 cyc./sec. Increase of the cycle frequency only intensified the fall in arterial blood pressure (Fig. 1).

In other experiments the duration of the stimulus was varied (0·05–2·0 msec.) frequency of discharge and voltage being kept constant (100 cyc./sec. and 2 V.). In each case a fall of blood pressure resulted from stimulation of the carotid sinus nerve (Fig. 2). This is in contrast to our previous observations in cats (Neil, *et al.* 1948, 1949). These experiments were repeated using rabbits under nembutal anaesthesia. The results were in no way different from those already described. Moreover, the intravenous injection of 0·1 g. of chloralose into such animals did not modify the blood-pressure response to sinus-nerve stimulation.

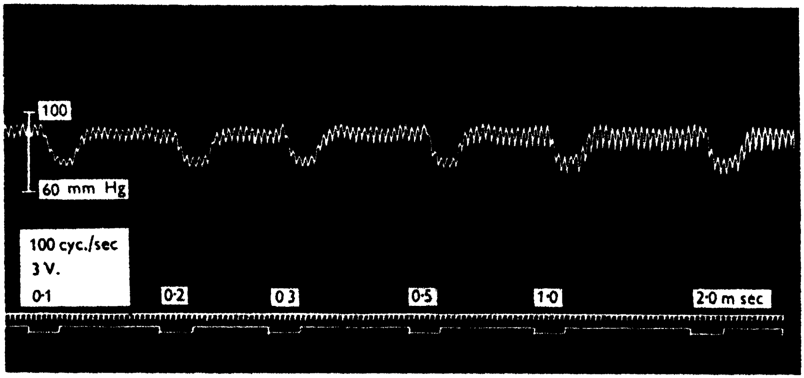

Fig. 2. Rabbit under chloralose anaesthesia. Records from above downwards: blood pressure, time in 5 sec., signal. Stimulation of right carotid sinus nerve with 3 V., 100 cyc./sec. and 0·1, 0·2, 0·3, 0·5, 1·0 and 2·0 msec. pulse duration. Note absence of pressor responses.

Electrical stimulation of the carotid sinus nerve in dogs. In dogs under chloralose anaesthesia, stimulation of the carotid sinus nerve, using the rectangular wave stimulator or induction coil, invariably produced a fall in arterial blood pressure. This response was obtained with all frequencies of stimulation used (10–100 cyc./sec.) and in no instance did a change in the pulse duration of the stimuli (0·05–1·0 msec.) affect the direction of the blood-pressure response (Fig. 3).

Similarly, stimulation of the carotid sinus nerve in dogs under nembutal anaesthesia produced a fall in arterial blood pressure, irrespective of the frequency and pulse duration of the stimuli, providing that threshold values of 10 cyc./sec. pulse frequency and 0·05 msec. pulse duration were exceeded (Fig. 4).

Neil *et al.* (1948, 1949) have previously reported that intravenous injection of chloralose into nembutalized cats causes a reversal of the depressor response to carotid sinus-nerve stimulation, marked rises of blood pressure being thus obtained.

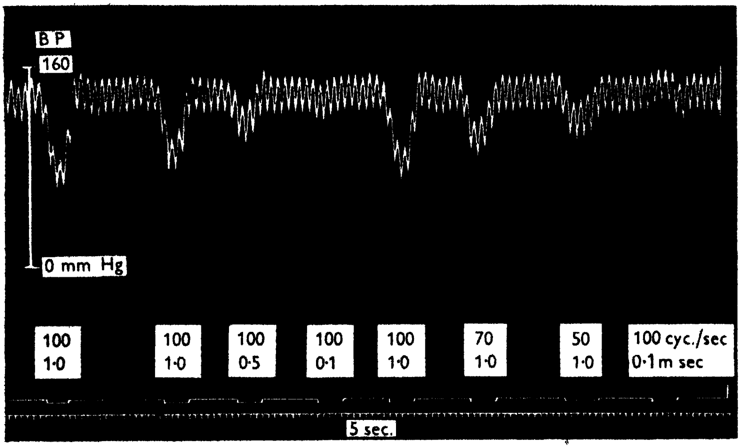

Fig. 3. Dog under chloralose anaesthesia. Records from above downwards: blood pressure, signal, time in 5 sec. Both vagi cut, left carotid sinus denervated. Right carotid sinus nerve stimulated with constant voltage 2 V. and varying pulse duration and frequency.

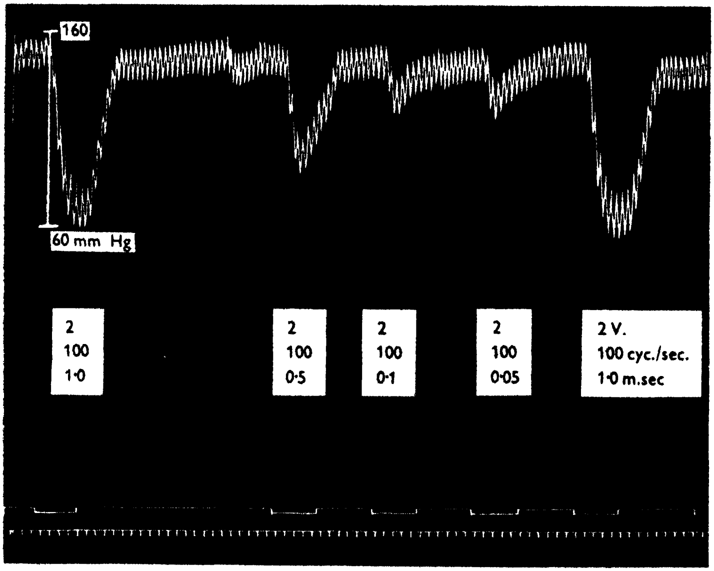

Fig. 4. Dog under nembutal anaesthesia. Records from above downwards; blood pressure, signal, time in 5 sec. Left carotid sinus denervated. Stimulation of right carotid sinus nerve with 2 V. 100 cyc./sec., and 1·0, 0·5, 0·1, 0·05 and 1·0 msec. pulse duration. Extent of blood-pressure fall varies with pulse duration.

These experiments were repeated in dogs. Fig. 5*a* shows typical results of responses to carotid sinus-nerve stimulation, using stimuli of varying pulse durations with constant frequency and voltage in a dog under nembutal anaesthesia. Fig. 5*b* was obtained from the same animal, 30 min. after the intravenous injection of 0·3 g. of chloralose. The responses to stimulation of the sinus nerve using identical variations of pulse duration can be seen to be unchanged.

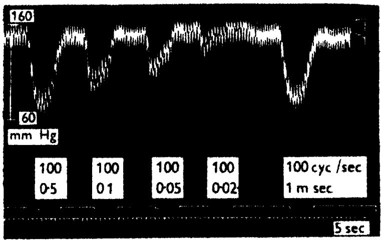

Fig. 5*a*.

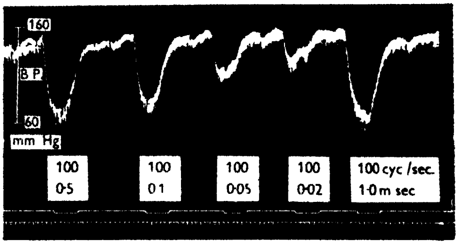

Fig. 5*b*.

Fig. 5*a*. Dog under nembutal anaesthesia. Records as in Fig. 4. Stimulation of right carotid sinus nerve with 2 V., 100 cyc./sec. and 0·5, 0·1, 0·05, 0·02 and 1·0 msec. pulse duration.

Fig. 5*b*. Continuation of Fig. 5*a*. 30 min. after intravenous injection of 0·3 g. of chloralose. Stimulation of right carotid sinus nerve with 2 V., 100 cyc./sec., and 0·5, 0·1, 0·05, 0·02 and 1·0 msec. pulse duration. Note absence of pressor responses.

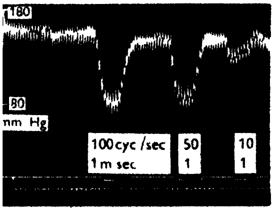

Fig. 6*a*.

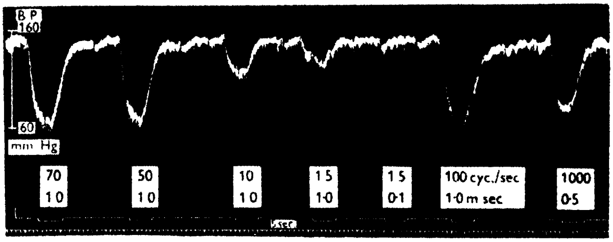

Fig. 6*b*.

Fig. 6*a*. Dog under nembutal anaesthesia. Records as in Fig. 4. Stimulation of right carotid sinus nerve with 2 V., 1·0 msec. pulse duration and 100, 50 and 10 cyc./sec.

Fig. 6*b*. Continuation of Fig. 6*a*. 30 min. after intravenous injection of 0·3 g. of chloralose. Stimulation of right carotid sinus nerve with 2 V. and 70, 50, 10 and 1·5 cyc./sec. with 1·0 msec. pulse duration, followed by 1·5 cyc./sec. and 0·1 msec., 100 cyc./sec. and 1·0 msec. and 1000 cyc./sec. and 0·5 msec. pulse duration. No evidence of pressor response.

Figs. 6*a*, *b* show the responses to sinus-nerve stimulations using stimuli of varying frequencies with constant voltage and pulse duration, in a nembutalized dog, before and 30 min. after the intravenous injection of 0·3 g. chloralose. Again the blood-pressure responses to stimulation of the nerve are unaltered.

DISCUSSION

The carotid sinus nerve trunk contains afferent fibres from both the baroceptor and chemoceptor regions of the carotid bifurcation area. It is well known that excitation of the baroceptor mechanism by increasing the intrasinusal pressure produces a marked fall in the arterial blood pressure. Furthermore, activation of the chemoceptors by changes in blood composition causes a rise in blood pressure. Results of electrical stimulation of the nerve trunk, however, using stimuli of well-defined characteristics, have not been previously reported.

In cats, under chloralose anaesthesia, the authors have previously shown that electrical stimulation usually causes a rise in arterial blood pressure. This is in contrast to the results of such stimulation in decerebrate cats or in cats under nembutal anaesthesia in which depressor responses are obtained. It was further shown that injection of chloralose in decerebrate or nembutalized cats reverses such depressor responses. Analysis of these phenomena showed that chloralose diminished the sensitivity of the vasomotor centre to baroceptor stimulation in addition to decreasing the excitability of the baroceptors themselves. Stimulation of the nerve trunk was thus considered to produce a rise of blood pressure by activating the chemoceptor component.

The present investigation has been carried out in order to see whether similar findings could be obtained in rabbits and dogs. No evidence has been found, in these animals that chloralose causes any modification of blood-pressure response to carotid sinus-nerve stimulation. In all circumstances a depressor response to electrical stimulation of the sinus nerve was obtained, indicating that the effects of baroceptor fibre stimulation are predominant over those of chemoceptor fibre stimulation. It was not possible to differentiate between the baroceptor and chemoceptor components of the sinus-nerve trunk by varying the pulse duration or pulse frequency of the stimuli used.

This interesting species variation may possibly be related to the marked anatomical difference in the configuration of the carotid bifurcation between the cat on the one hand and the dog and rabbit on the other. In the cat, the internal carotid artery is extremely small and functionally unimportant (Davis & Story, 1943). In the dog and rabbit, however, the internal carotid artery is of notable size and would appear to be of greater functional importance.

SUMMARY

1. Electrical stimulation of the carotid sinus-nerve trunk in rabbits and dogs under chloralose and nembutal anaesthesia, using a rectangular wave electronic stimulator or an induction coil, produced marked depression of the arterial blood pressure.

2. Variation in frequency and in pulse duration of the stimuli did not alter the direction of the blood-pressure response.

3. Injection of chloralose into nembutalized rabbits and dogs did not modify the nature of the blood-pressure response to electrical stimulation of the sinus nerve.

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THE NATURE OF THE ACTION OF RENIN AND HYPERTENSIN ON RENAL FUNCTION IN THE RABBIT

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In a previous paper (Pickering & Prinzmetal, 1940) it was shown that renin has a remarkable effect on urine flow in the rabbit. After intravenous injection the flow of urine was reduced during the first 10 min., and then rose to very high levels, as much as 50 ml. being produced in a half-hour period. The glomerular filtration rate, as measured by creatinine clearance, was found to fall during the antidiuretic phase, and to return to normal during the period of diuresis. The urine formed during the diuretic phase was remarkable in that its content of sodium and chloride tended to approximate closely to, and in the case of chloride slightly to exceed, that of plasma; and this was true whether the initial values of chloride were high or low. On the other hand, urea excretion was not dissimilar to that observed in a water diuresis of comparable degree. Since that work was done it has been shown that renin has the properties of an enzyme, acting on a constituent of the plasma globulins (hypertensinogen, or renin substrate) to form a substance of small molecular size, hypertensin (Braun-Menendez, Fasciolo, Leloir & Munoz, 1940) or angiotonin (Page & Helmer, 1940). As far as vascular effects are concerned, the action of renin seems to be entirely due to the formation of hypertensin; the actions of the two are qualitatively similar, and in the absence of hypertensinogen renin has no action on the blood pressure or the vessels of the perfused rabbit's ear (Page & Helmer, 1940). It therefore seemed of interest to inquire whether hypertensin would produce effects on renal function in the rabbit similar to those produced by renin. The opportunity has also been taken to study more closely the mode of action of these substances.

METHODS

The methods were essentially similar to those used in a previous publication (Pickering & Prinzmetal, 1940). Urine was obtained by catheter at intervals usually of 30 min. from unanaesthetized male rabbits (2 to 2·5 kg.) which were lightly held on their backs on the lap, the bladder being washed out with 5 ml. water in each instance. The room in which the experiments were done was kept warm (25–26°) to ensure dilatation of the ear vessels. All injections were given into, and all blood samples taken from, the left ear which had had its sensory nerves cut by previous aseptic operation; for these purposes the rabbit was taken from its cage and placed on a warm pad on which it was lightly secured with a cloth. Between catheterizations, the animals were placed in small wire cages in which they could turn round with difficulty. In these experiments urine was not voided naturally between the catheterizations. The rabbits were fed on a mixed diet, starved for 12 hr. previously but allowed access to water. Unless otherwise stated, all the experiments in this paper were during the subsidence of water diuresis induced by giving 100 ml. water by stomach tube some 3 hr. before urine collections were begun.

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Renin and hypertensin. Renin was prepared from rabbit's kidney by a method previously described (Pickering & Prinzmetal, 1940). Alcohol-dried kidney powder was extracted with saline, clarified by acidifying to pH 5 and centrifuging, and the supernatant fluid half saturated with $(NH_4)_2SO_4$. The precipitate was dialysed till free of sulphate and the solution dried by the lyophil process. The yellow powder, completely soluble in saline, was used as required.

Hypertensin was prepared from ox hypertensinogen and rabbit renin by a method based on that of Braun-Menendez *et al.* (1940). Ox serum was half saturated with $(NH_4)_2SO_4$. The precipitate, separated by centrifuging, was dialysed till free of sulphate and filtered. Hypertensinase was destroyed by acidifying to pH 4·2 for 20 min. at 25° C. After neutralizing, the hypertensinogen solution was brought to 37° C., and to it was added an amount of renin previously found in pilot experiments to give a maximum yield of hypertensin. An amount of renin corresponding to 10 g. fresh rabbit's kidney was used for the hypertensinogen derived from 300 ml. ox serum. After incubating for 20 min., the mixture was poured into 4 vol. of a warm mixture of 9 vol. ethanol and 1 vol. ether, and filtered. The filtrate was distilled *in vacuo* at 50° C., and finally evaporated to dryness on a water-bath in air. The brown residue was taken up in saline to give a turbid solution which was clarified by shaking with ether. After separating, the watery layer was freed from ether on the water-bath. In some preparations a further precipitation with 4 vol. of absolute alcohol was carried out, and the filtrate evaporated to dryness and taken up again in normal saline.

Controls for hypertensin were prepared in the following way at the same time as two of these preparations. After destroying hypertensinase, a portion of the hypertensinogen was put through the process just described save that no renin solution was added before incubating at 37°. The final solution was made up to a volume which bore the same relation to the volume of the original hypertensinogen solution as did the final volume of the hypertensin preparation. Thus the control (hypertensin control) should have contained all the substances in the same proportions as did the hypertensin solution, except hypertensin itself.

Inulin and diodone clearances. 1 g. of purified inulin in 10 ml. saline, and 5 ml. of a 35% solution of diodone were injected subcutaneously in the back and flanks just before, or just after, the administration of water. Clearances were done 4–6 hr. later, although in some cases after 6 hr. the plasma diodone had fallen too low for reliable results. Heparinized blood samples were obtained before injection of inulin and diodone, and in the middle of each period of urine collection. Both blood and urine samples were analysed by the methods of Hubbard & Loomis (1942) and Alpert (1941) with a slight modification in the case of diodone (Sanderson, 1948). The methods of estimating sodium and chloride were those of Morton (1945) and Sendroy (1937) respectively.

Glucose Tm. In male rabbits anaesthetized with intravenous nembutal (sodium pentobarbitone) a priming dose of 20 mg./kg. body weight creatinine and 0·9 g./kg. glucose in 6 ml. water was given intravenously, followed by a continuous intravenous infusion. In the first four experiments of Table 4, a solution containing 2·2 g. glucose and 70 mg. creatinine per kg. body weight in 100 ml. water was infused at a rate of 80 ml./hr. In the last two experiments of Table 4 a solution of creatinine and glucose four times as strong was given at 20 ml./hr. A catheter introduced into the bladder per urethram was tied in; urine was obtained at 10 min. intervals, the bladder being washed out with 5 ml. water and 5 ml. air. Heparinized blood samples were obtained from the dilated ear veins in the middle of each clearance period. Clearances were not begun until at least 25 min. after the infusion had begun.

Berlin blue injection of kidneys. The rabbit was anaesthetized with nembutal and tied on a table. The belly was opened ventrally, the abdominal aorta tied at its bifurcation and the left renal pedicle carefully exposed without touching the vessels. 10 ml. 2% soluble Berlin blue were injected in 10 sec. through a wide needle into the aorta just headwards of the ligature. As the last 2 c.c. were being injected the left renal pedicle was abruptly clamped in artery forceps, the ureter was next clamped and the kidney excised with the clamps attached. After fixing in formalin, transverse sections cut freehand at about 0·5 mm. were cleared in methyl salicylate and observed under the binocular microscope. Stained sections prepared from paraffin blocks were also examined. In four rabbits renin was injected intravenously after anaesthetization; 10 min. later the belly was opened and the aorta exposed ready for injection. In all these experiments the ureters were widely dilated with urine and the bladder filled quickly. It may be assumed, therefore, that at the time of injection renin was exerting its customary diuretic effect.

RESULTS

The effect of hypertensin. In a given rabbit the rise of arterial pressure produced by intravenous injection of hypertensin is, as with renin, roughly related to the logarithm of the dose. With both renin and hypertensin, however, the quantitative relationship differs from rabbit to rabbit. We have not attempted in these experiments to define the dosage in terms of arbitrary units, but we have adjusted the dose of the four preparations of hypertensin used so that they gave the same order of response, namely rises of arterial pressure varying from 25 to 50 mm. Hg in the different rabbits. With such doses the rise reaches its peak in 35–60 sec. and lasts 2–5 min. (see Fig. 1). As with renin, and in contrast to adrenaline and tyramine, responses of this order are unaccompanied by macroscopic change in the ear vessels of the warm rabbit.

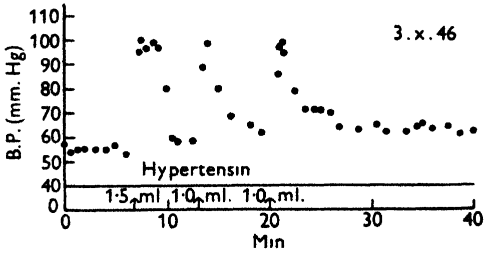

Fig. 1. Effect of the doses of hypertensin here employed on the unanaesthetized rabbit's blood-pressure. The rabbit and hypertensin preparation were the same as in Fig. 7; the time 24 hr. later.

To determine whether hypertensin has a diuretic effect, it was injected towards the end of the falling limb of the curve of water diuresis. Urine collections were begun about 3 hr. after the hydrating dose of water, and continued for at least three half-hour periods, and until it was clear that the rate of urine formation was decreasing and until the volume per half-hour period was less than 10 ml. In these circumstances it is the rule for diminishing rate of water excretion to be accompanied by rising urinary chloride percentage, the rate of excretion of chloride remaining nearly constant (Figs. 6–8). Hypertensin was then injected intravenously in three doses at 3 min. intervals and the half-hourly collections continued. In all sixteen such experiments on ten rabbits and using three separate preparations of hypertensin, the volume of urine increased during the half-hour after the injection of hypertensin, the increase continuing into the next half-hour in five rabbits. The urine volume in the third half-hour after injection was less than the pre-injection level in all. These experiments are summarized in Table 1. As with renin a very conspicuous rise in chloride excretion accompanied the diuresis, this being due chiefly to increase in the urinary chloride concentration in these experiments in which this was initially low, for in these animals no sodium chloride above that contained in the diet had been given by mouth (Fig. 2).

TABLE 1. Urine flow and chloride excretion in five successive half-hour periods of which two (1, 2) preceded and three (3, 4, 5) followed intravenous injection of hypertensin and hypertensin control

	Before hypertensin		After hypertensin		
	1	2	3	4	5
(1) Rabbits not previously given salt, ten experiments, nine rabbits					
Urine flow (ml./min.)	0·18	0·135	0·37	0·10	0·035
Cl′ excretion (μequiv./min.)	5·2	3·9	40·5	10·8	3·3
(2) Rabbits previously given salt, six experiments, six rabbits					
Urine flow (ml./min.)	0·21	0·17	0·56	0·25	0·06
Cl′ excretion (μequiv./min.)	35	32	88	46	16·8
(3) Control experiment, no salt, five experiments, five rabbits					
	Before control		After control		
Urine flow (ml./min.)	0·31	0·24	0·15	0·11	0·10
Cl′ excretion (μequiv./min.)	5·5	5·2	5·1	5·0	—

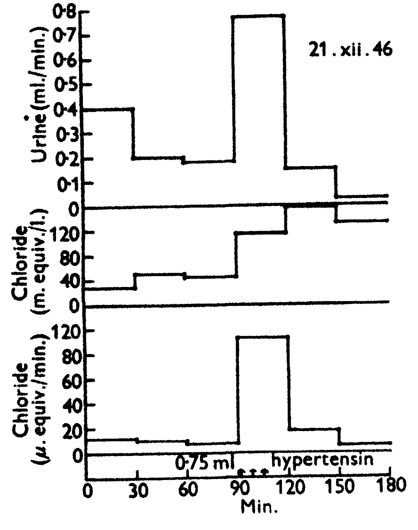

Fig. 2. Rabbit 478, 21 December 1946. Effect of three intravenous injections of 0·35 ml. hypertensin at 4 min. intervals on the rate of urine flow (top line), urinary chloride content (second line) and the urinary chloride excretion (bottom line) over the successive half-hour periods. The rabbit received 100 ml. H_2O by stomach tube 5 hr. before the figure begins.

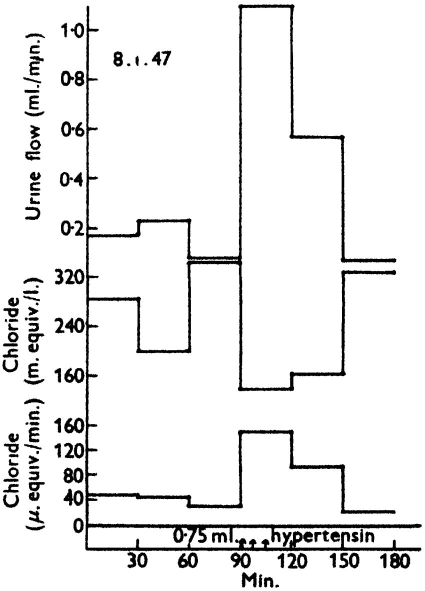

Fig. 3. Rabbit 486, 8 January 1947. Effect of hypertensin when chloride excretion was initially high. The rabbit had received 100 ml. of 1·5 % solution of sodium chloride the evening before and again 5 hr. before the figure begins.

A similar effect on chloride excretion following the injection of renin had suggested that the phenomenon was due to an inhibition of chloride reabsorption with consequent increase in the excretion of water by osmotic action. This was tested and disproved by greatly raising the initial urinary chloride levels (Pickering & Prinzmetal, 1940). In this respect also, the action of hypertensin resembles that of renin. In order to raise the initial urinary chloride concentration, 100 ml. of 1·5% sodium chloride were given by stomach tube the evening before and again on the morning of the experiment, hypertensin being injected 4–5 hr. later. When the urinary chloride exceeded 180 m.equiv./l. hypertensin reduced the urinary chloride concentration, but produced the usual rise of urine output and of total chloride excretion (Table 1 and Fig. 3).

To ensure that these responses were due to hypertensin and not to an impurity present in our solutions, 'hypertensin control', prepared as previously described, was injected intravenously in similar amounts. This control preparation had no significant effect on the rabbit's blood pressure and, as Table 1 shows, was without influence on the rate of formation of urine, or its chloride content. It may be inferred, therefore, that the effects on the flow and chloride content of urine previously described were due to hypertensin itself.

As with renin, the diuretic effect seems to occur after the main pressor effect is over. In the case of renin, where the responses to a single injection are long drawn out, the initial phase of greatest rise of blood pressure was found associated with a reduced urine flow. Diuresis occurred later, often continuing, and sometimes being greatest, in the second half-hour when the arterial pressure had nearly or quite returned to normal. The response to hypertensin is briefer, and therefore more difficult to analyse in respect of time. We made no collections in periods shorter than 30 min. and never saw striking diuresis in the second half-hour period. Chloride excretion remained relatively constant over the separate half-hour collecting periods before hypertensin injection and changes were therefore more easily recognized. In rather more than half our experiments chloride excretion was still definitely raised in the second half-hour at a time, when, after similar doses, the rise of arterial pressure had subsided.

Comparison of hypertensin with tyramine and adrenaline. In a previous publication (Pickering & Prinzmetal, 1940) it was stated that tyramine acid phosphate produced no consistent increase in urine flow or chloride excretion. Renewed inspection of these data revealed a definite tendency to an increase in both these values, which was, however, trivial as compared with the effects produced by renin. It therefore seemed of some interest to compare the effects of hypertensin, tyramine and adrenaline, in doses having a similar pressor effect. The comparison was made on six rabbits using doses of 0·25 ml. hypertensin (preparation 465), 3 mg. tyramine acid phosphate (in 0·5 ml. saline) and 0·4 mg. adrenaline tartrate (in 0·4 ml.). These doses gave pressor responses of the same order. Thus the average response to hypertensin was 42 mm. and its duration 3½ min., to tyramine 46 mm. and 3½ min., and to adrenaline 46 mm.

and 1½ min. Two sets of experiments were done. In one the animals were hydrated with tap water; these rabbits had a low excretion of chloride. In the other, the animals were given 100 ml. of 1·5% sodium chloride by stomach tube the previous evening and deprived of water and food until the morning when they were again given 100 ml. of 1·5% sodium chloride instead of the usual hydrating dose of water; these animals had a high initial rate of chloride excretion. Each of the three substances was injected intravenously in three equal doses of the amounts mentioned at 3 min. intervals at the beginning of what is termed the first half-hour collecting period after administration. The results on urine flow are summarized in Table 2. Hypertensin produced an increase in urine flow in all eleven experiments, tyramine in nine out of eleven, and adrenaline in five out of six. When the urinary chloride was low, all three substances produced a rise both in the chloride percentage and in the absolute excretion in the urine, but this was most pronounced with hypertensin. When the chloride percentage was high, all three substances produced an increased excretion of chloride, with a tendency for the chloride percentage in the urine to fall. With minor exceptions the average figures presented in Table 2 do not reveal any pronounced difference between the action of the three substances, which, in these doses, have a diuretic and chlorouretic action. Detailed inspection of the results reveals, however, that there is a striking difference between them in the extent to which they influence the chloride content of the urine in the post-injection period. This is shown in Fig. 4, which compares the chloride percentages in the urine collected in the successive periods immediately before and after injection of the substances. It will be seen that while in each case there is a tendency for the chloride content of the urine to approximate to a mean position, this tendency is feeble in the case of tyramine and adrenaline, but pronounced after hypertensin. Data from experiments previously reported (Pickering & Prinzmetal, 1940) show that in this respect hypertensin closely

TABLE 2. A comparison of the effects of hypertensin, tyramine, and adrenaline on urine flow and chloride excretion

Substance injected	No. of rabbits	Urine flow (ml./min.)					Chloride excretion (μequiv. Cl′/min.)				
		Before		After			Before		After		
		1	2	3	4	5	1	2	3	4	5
After hydrating dose of 100 c.c. H_2O 5–7 hr. before											
Hypertensin	5	0·17	0·13	0·24	0·10	0·04	5·2	4·4	31·0	10·2	5·0
Tyramine	5	0·15	0·13	0·22	0·10	0·05	1·4	1·6	6·2	1·9	1·6
Adrenaline	3	0·27	0·24	0·29	0·17	0·12	8·0	8·0	12·4	—	—
After 100 c.c. 1·5% NaCl 5–7 and 21–23 hr. before											
Hypertensin	6	0·21	0·17	0·56	0·25	0·05	37	34	90	48	16·8
Tyramine	6	0·25	0·18	0·32	0·08	0·06	35	30	51	22	18
Adrenaline	3	0·16	0·10	0·52	0·11	0·06	35	22	65	27	14

resembles renin. In fact during the diuresis following the intravenous injection of renin or hypertensin, the concentration of chloride in the urine approximates to a value rather greater than that of plasma, and this occurs whether the urinary chloride was initially much below or much above that level.

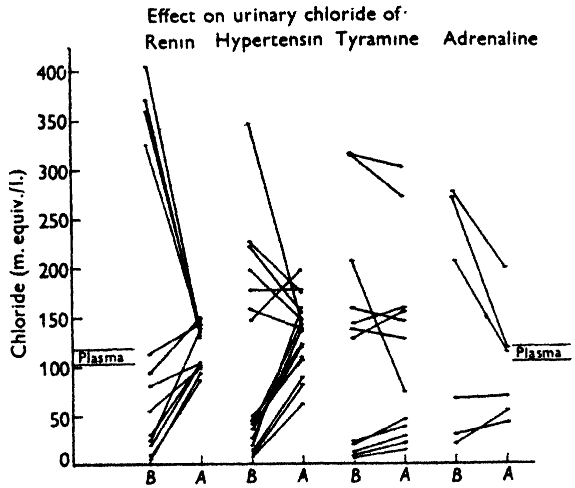

Fig. 4. Effect of renin, hypertensin, tyramine and adrenaline on the urinary chloride. Each line represents the change in urinary chloride from the period before (*B*) to the period after (*A*) the administration of the substance. The data for hypertensin, tyramine and adrenaline are from the experiments summarized in Table 2 (plus three additional experiments with the same dose of hypertensin), and the data for renin those obtained in a previous paper (Pickering & Prinzmetal, 1940).

The effect of renin and hypertensin on inulin and diodone clearances. The qualitative similarity between the effects of hypertensin, tyramine and adrenaline on the volume and composition of the urine in the unanaesthetized rabbit suggest that these effects may be vascular in origin. To investigate this further we have measured the renal clearances of inulin and diodone over half-hour periods before, immediately after, and an hour or more after the intravenous injection of renin and hypertensin. The results are summarized in Table 3 which includes data for the periods only in which these clearances were measured, the urine flows and chloride contents for the other periods being omitted.

Five experiments were done in which a single dose of renin was injected at the beginning of a collecting period. In all, a diuresis and a conspicuous increase in the urinary excretion of chloride occurred, and in the two experiments where this was measured a concomitant increase in the excretion of sodium. In two experiments the increase in urine volume and chloride excretion was greatest in the second half-hour, and this was true in two other similar observa-

TABLE 3. The effect of renin and hypertensin on inulin and diodone clearances in the rabbit

Date and rabbit	Period	Urine flow (ml./min.)	C_{IN} (ml./min.)	C_D	F.F. (%)	Urine (µequiv./min.) Na	Urine (µequiv./min.) Cl	Urine (m.equiv./l.) Na	Urine (m.equiv./l.) Cl	Serum (m.equiv./l.) Na	Serum (m.equiv./l.) Cl
24. ix. 46 478	2.47–3.17	0·12	5·0	30·9	16·1	1·15	2·8	9·6	23·3	150	—
	3.20	3·0 ml. (7·5 mg.) renin									
	3.17–3.45	0·25	5·4	21·5	25·1	23·5	27·0	94·0	108·0	149	—
	3.45–4.16	0·08	5·3	26·8	19·7	10·2	12·6	127·0	156·3	148	—
26. ix. 46 479	1.52–2.22	0·081	6·2	35·5	17·4	2·8	8·8	33·0	109·0	140	108
	2.25	3·0 ml. (9 mg.) renin									
	2.22–2.52	0·216	7·1	27·6	25·7	16·8	27·6	78·0	128·0	144	106
	2.52–3.22	0·37	7·1	36·3	19·6	44·4	52·2	120·0	141·0	140	105·5
	4.24–4.58	0·10	7·3	—	—	—	5·7	—	57·0	—	105
26. viii. 46 472	2.00–2.30	0·38	6·4	32·2	19·9	—	6·0	—	15·8	—	—
	2.40	3 ml. (9 mg.) renin									
	2.30–3.00	0·26	4·9	16·6	29·5	—	22·7	—	87·2	—	—
	3.00–3.30	0·43	5·7	23·4	24·4	—	67·5	—	157	—	—
	3.30–4.00	0·03	4·1	19·0	21·6	—	5·4	—	179	—	—
29. viii. 46 477	3.30–4.00	0·017	8·0	34·7	23·0	—	3·0	—	176	—	—
	4.04	4 ml. (12 mg.) renin									
	4.00–4.30	0·30	6·7	21·1	31·8	—	33·0	—	110	—	—
	5.00–5.30	0·033	6·0	31·8	18·9	—	1·61	—	48·7	—	—
4. ix. 46 478	3.30–4.02	0·033	5·8	31·8	18·3	—	1·5	—	45·6	—	—
	4.04	2 ml. (6 mg.) renin									
	4.02–43.2	0·42	5·8	19·5	29·8	—	50·4	—	120	—	—
	5.00–53.0	0·017	6·1	34·0	18·0	—	0·27	—	15·7	—	—

1. viii. 46	2.50–3.20	0·27	8·1	—	—	9·7	14·7	36	54·5	139	104
474	3.52	3 ml. hypertensin (1 ml.–40 mm. Hg)									
	3.52–4.25	0·755	7·1	—	—	68·2	60·5	90	80	142	106·7
	4.58–5.25	0·036	6·0	—	—	2·4	2·62	66	73	142	108·6
2. x. 46	3.03–3.35	0·20	7·7	42·1	18·3	1·38	5·4	6·9	27	144	—
479	3.37–3.49	1·5 ml,. 1·0 ml., 1·0 ml. hypertensin									
	3.35–4.06	0·20	7·8	30 4	25·7	19·8	28·4	99	142	147	—
	4.34–5.07	0·09	7·1	33·8	21·0	6·6	11·4	74	127	—	—
12. viii. 46	3.10–3.39	0·53	7·0	34 5	20·3	—	8·0	—	15	—	113
472	4.11	2·5 ml. hypertensin									
	4.08–4.38	0·65	8·0	27·1	29·5	—	52·0	—	80	—	112
	5.08–5.38	0·033	7·4	34·6	21·4	—	2·3	—	69·6	—	114
13. viii. 46	3.40–4.10	0·116	7·8	40·2	19·4	—	3·25	—	28	—	—
473	4.10–4.17	1·0 ml. 1·5 ml. 1·0 ml. hypertensin									
	4.10–4.40	0·05	6·3	26·7	23·6	—	4·5	—	90	—	—
	5.10–5.40	0·033	8·4	44·0	19·1	—	1·65	—	50	—	—
13. viii. 46	3.45–4.15	0·127	8·4	45·7	18·4	—	2·69	—	21·2	—	108
475	4.49–4.58	1·5 ml. 1·5 ml. and 1·5 ml. hypertensin									
	4.48–5.15	0·52	3·8	13·0	29·2	—	76·4	—	147	—	124
	5.15–5 45	0·95	4·9	16·2	30·2	—	144·0	—	152	—	—
	5.45–6.15	0·15	3·1	16·4	18·9	—	24·0	—	160	—	115
2. x. 46	3.00–3.31	0·14	6·0	34·6	17·3	1·37	5·1	9·8	36·2	147	—
478	4.02–4.18	1·0 ml. 1·5 ml. and 1·0 ml. hypertensin control									
	4.00–4.31	0·12	6·6	37·3	17·7	2·04	6·4	17	53·2	147	—
	4.31–5.03	0·039	6·2	30·0	20·7	2·24	6·2	57·5	158	145	—

tions on one of these rabbits, 479, in which clearances were not measured. Renin produced no constant or striking change in inulin clearance measured over the half-hour period, a result confirming that earlier obtained for creatinine clearance (Pickering & Prinzmetal, 1940). Diodone clearance was always reduced significantly in the first half-hour period after renin, and the filtration fraction was conspicuously raised. In rabbit 479, diodone clearance and filtration fraction had returned to normal in the second half-hour period in which the increase in urinary volume and chloride was most pronounced (Fig. 5). In rabbit 472, however, in which the greatest increase in urinary volume and chloride also occurred in the second half-hour, diodone clearance and filtration fraction had not returned to their pre-injection level. In the third half-hour both inulin and diodone clearances were low, though the filtration fraction was normal, a result which may have been due to incomplete collection of urine, though it seems from our data that this rarely occurred.

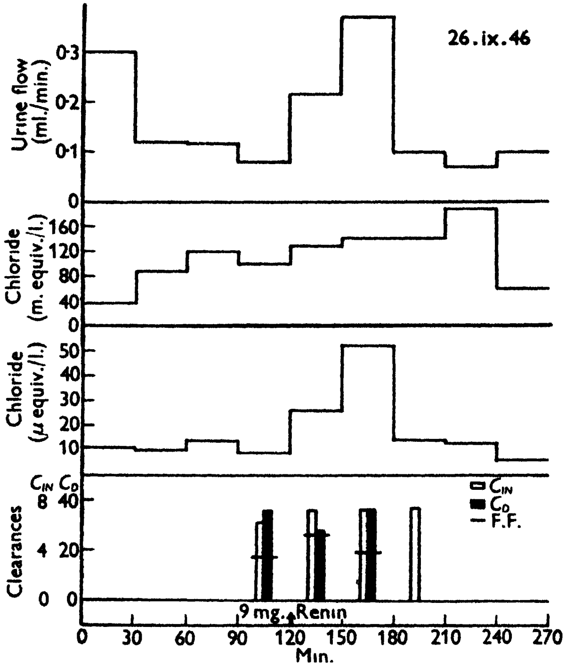

Fig. 5. Effect of 3 ml. renin on urine flow, chloride excretion, inulin and diodone clearances and the filtration fraction. Rabbit (479) unanaesthetized. In this and the two subsequent figures the scale for filtration fraction (*FF*) is that for diodone clearance (*CD*).

The effects of hypertensin on the clearances were in general similar to those of renin. During the collecting period at the beginning of which the injections were given, diodone clearance always fell significantly and the filtration fraction was increased. Changes in inulin clearance were variable and, with one exception, slight. An hour after the injection inulin and diodone clearances and filtration fraction had returned to normal or nearly so in all experiments except one. The exceptional rabbit (475) responded with a very large diuresis, particularly in the second half-hour, the urinary chlorides rising to above plasma level in both periods, and it developed signs of acute pulmonary oedema after the third injection of hypertensin. Inulin clearance was significantly reduced during both periods of large urinary outputs and remained low during subsequent half-hour collecting periods in which the diuresis and the increased chloride excretion had subsided (Fig. 6). So far as we could ascertain this response was due to hypertensin, because subsequent injections of smaller doses led to no abnormal symptoms, and the control preparation injected subsequently in similar amounts was without effect on urinary chloride or output. The effects on clearances and filtration fraction in the rabbit are similar to those which others have reported with both renin and hypertensin in man and the dog, though in neither species has a similar effect on urinary chloride and output been described.

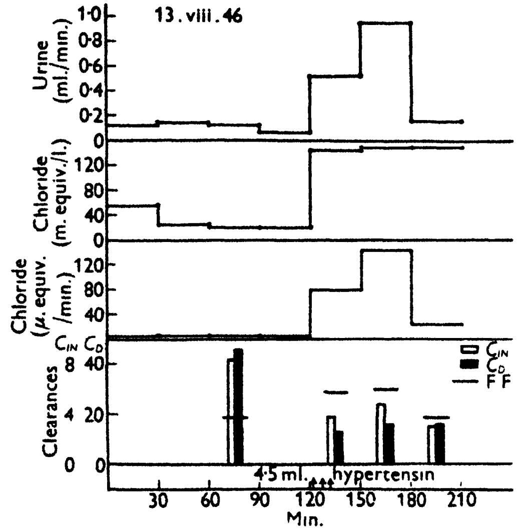

Fig. 6. Very intense diuresis to hypertensin with pronounced effects on urinary chloride, and the clearances. Rabbit (475) unanaesthetized.

With renin, difficulty had been experienced in obtaining satisfactory diureses when the interference inherent in blood sampling was added. To ensure a diuretic response it proved to be necessary to obtain blood samples from an ear previously desensitized by nerve section (Pickering & Prinzmetal, 1940). It was also found with renin that the diureses obtained under such conditions were less pronounced than when no blood samples were taken. Even when the ear is insensitive, blood sampling, necessitating as it does the animal being restrained for 3 or 4 min. while its ear is manipulated, produces a change in the experimental conditions. It is to this that we attribute the failure of diuresis to occur during the period at the beginning of which hypertensin was injected in rabbits 473 and 479. Each of these animals did, however, show the usual changes in urinary chloride, large in 479 (Fig. 7), small in 473.

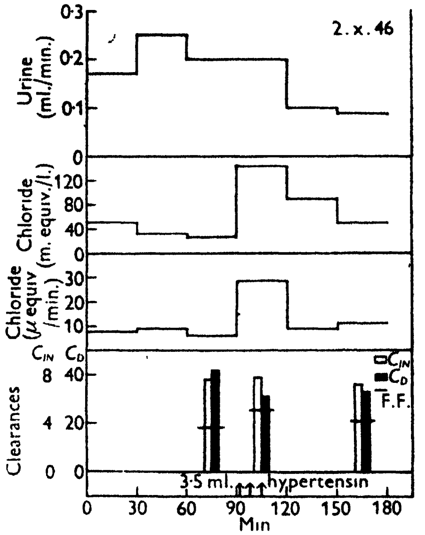

Fig. 7. Effect of hypertensin on glomerular filtration rate, diodone clearance, filtration rate, and chloride excretion; no diuresis. Rabbit (479) unanaesthetized.

The action of renin on the tubular reabsorption of glucose. A rise in the effective head of pressure in the glomeruli leads to an increased excretion of water and salt in both the isolated kidney (Richards & Plant, 1922; Starling & Verney, 1925) and anaesthetized animal (Eggleton, Pappenheimer & Winton, 1940), presumably because of the more rapid flow of urine along the tubules. The effects of renin and hypertensin might well be due to such a mechanism for both substances raise arterial pressure, and the fraction of plasma filtered in traversing the glomeruli. If the number of functioning nephrons remained unchanged, an increased flow along the tubules could only be achieved by a greatly increased glomerular filtration rate. But the inulin clearances show that the glomerular filtration rate is not necessarily increased when urine flow and urinary chloride are raised by hypertensin or renin. It seems, therefore, that if we are dealing with a pressure diuresis it must be one in which a reduced number of nephrons participate. To see whether this were so, we determined the capacity of the tubules to reabsorb glucose (glucose Tm, Smith, 1939) at blood glucose values above 600 mg./100 c.c., using creatinine clearance as a measure of glomerular filtration rate. These experiments were performed under nembutal anaesthesia to allow the maintenance of adequate plasma glucose and creatinine levels by intravenous infusion of the substances. Fig. 8 illustrates and Table 4 summarizes these experiments in which a single injection of two units of renin was given. After renin, urinary output rose above the pre-injection level in two experiments out of five in the first 10 min. and in five out of six in the second 10 min. Chloride excretion was increased in both first and second 10 min. periods in all four experiments where it was measured and particularly in the second 10 min.

Table 4. Effect of renin on creatinine clearance and tubular capacity to reabsorb glucose (glucose Tm)

(Successive 10 min. clearance periods are indicated by −3, −2, −1 before and +1, +2, +3 after injection of renin.)

		Urine flow (ml./min.)						Chloride excretion (μequiv./min.)					Creatinine clearance (ml./min.)				Glucose Tm (mg./min.)			
		Before renin			After renin			Before renin		After renin			Before renin		After renin		Before renin		After renin	
Rabbit	Date	−3	−2	−1	+1	+2	+3	−2	−1	+1	+2	+3	−2	−1	+1	+2	−2	−1	+1	+2
494	29. x. 47	0·13	0·21	0·4	—	0·95	1·35	—	—	—	—	—	10·1	11·3	—	9·2	29·9	33·5	—	31·7
487	25. x. 47	—	1·3	1·25	1·3	3·2	1·95	—	—	—	—	—	7·9	8·3	8·8	10·3	20·8	28·0	32·1	29·2
487	30. x. 47	—	0·73	0·68	1·36	2·4	2·16	30·5	13·5	61·0	204	140	6·4	6·9	7·9	8·2	20·6	22·0	23·7	24·3
486	5. xi. 47	0·95	0·75	0·88	0·76	1·76	1·21	33·0	32·0	37·0	104	64·0	6·7	7·4	6·6	8·9	25·3	29·4	30·1	36·9
494	6. xi. 47	2·14	1·45	1·58	1·32	1·33	0·65	11·0	4·47	57·8	73·0	73·6	11·7	13·4	12·4	10·2	29·1	36·5	35·8	29·1
486	13. xi. 47	0·27	0·2	0·24	0·41	0·91	—	29·1	19·0	56·5	69·9	—	6·5	6·7	6·5	6·9	18·4	21·0	22·1	25·9

TABLE 5. Effect of renin and hypertensin on sodium and chloride reabsorption

Exp. and date	Period	C_{IN} (ml./min.)	Urine flow (ml./min.)	Water (% excreted)	Chloride			Sodium		
					Glom. filtrate (µmol./min.)	Excreted (µmol./min.)	% excreted	Glom. filtrate (µmol./min.)	Excreted (µmol./min.)	% excreted
1	2.47–3.17	5·0	0·12	2·4	—	—	—	750	1·15	0·15
24. ix. 46	3.20	7·5 mg. renin in 3 ml.								
	3.17–3.45	5·4	0·25	4·6	—	—	—	850	23·5	2·9
	3.45–4.16	5·3	0·08	1·5	—	—	—	785	10·2	1·3
2	1.52–2.22	6·2	0·081	1·3	670	8·8	1·3	868	2·7	0·32
26. ix. 46	2.25	9 mg. renin in 3 ml.								
	2.22–2.52	7·1	0·216	3·0	753	27·6	3·7	1022	16·8	1·6
	2.52–3.22	7·1	0·37	5·2	748	52·2	7·0	994	44·4	4·5
	4.24–4.58	7·3	0·10	1·4	766	5·7	0·74	—	—	—
6	2.50–3.20	8·1	0·27	3·3	842	14·7	1·7	1126	9·7	0·86
1. viii. 46	3.52	3 ml. hypertensin								
	3.52–4.25	7·1	0·755	10·6	756	60·5	8·0	1008	68·2	6·8
	4.58–5.25	6·0	0·036	0·6	651	2·62	0·4	852	2·4	0·28
7	3.03–3.35	7·7	0·20	2·6	—	—	—	1109	1·38	0·12
2. x. 46	3.37–3.49	3·5 ml. hypertensin								
	3.35–4.06	7·8	0·20	2·6	—	—	—	1145	19·8	1·7
	4.34–5.07	7·1	0·09	1·3	—	—	—	1029	6·66	0·64
8	3.10–3.39	7·0	0·53	7·6	791	8·0	1·0	—	—	—
12. viii. 46	4.11	2·5 ml. hypertensin								
	4.08–4.38	8·0	0·65	8·1	896	52·0	5·8	—	—	—
	5.08–5.38	7·4	0·033	0·45	844	2·3	0·27	—	—	—
10	3.45–4.15	8·4	0·127	1·51	907	2·69	0·30	—	—	—
13. viii. 46	4.49–4.58	4·5 ml. hypertensin								
	4.48–5.15	3·8	0·52	13·7	471	76·4	16·2	—	—	—
	5.15–5.45	4·9	0·95	19·4	—	—	—	—	—	—
	5.45–6.15	3·1	0·15	4·8	356	24·6	6·75	—	—	—
11	3.00–3.31	6·0	0·14	2·3	—	—	—	882	1·37	0·16
2. x. 46	4.02–4.18	3·5 ml. hypertensin control								
	4.00–4.31	6·6	0·12	1·8	—	—	—	970	2·04	0·21
	4.31–5.03	6·2	0·039	0·63	—	—	—	899	2·24	0·25

The figures for sodium and chloride in the glomerular filtrate have been obtained by multiplying plasma Na and Cl by inulin clearance. No allowance has been made for Donnan equilibrium or for the space occupied by the plasma proteins.

The increased output of urine and of chloride effected by renin are not accompanied by any constant change in glomerular filtration rate or glucose Tm.

Appraisal of these results is complicated by the tendency, least marked in Fig. 8, of urinary output to rise during the period before injection of renin, a tendency no doubt resulting from the continuous intravenous infusion. As Kaplan & Smith (1935) and Dicker & Heller (1944–5) have shown, the rabbit is peculiar in that more nephrons are called into action as urinary output rises in water diuresis. Both creatinine clearance and glucose Tm were rising in the period immediately preceding renin injection, and we cannot exclude the possibility that any further rises in the periods following renin injection were due to a continuation of this response to increasing water load. There is, however, no difficulty in deciding from these figures whether or not renin diuresis is due to an increased rate of glomerular filtration per functioning nephron, which can be gauged by the ratio $\frac{\text{creatinine clearance}}{\text{glucose Tm}}$. Taking the two experiments (Exps. 2 and 4) with the largest diuretic response to renin, this ratio was in the two periods before renin 0·37 and 0·30, and in the two periods after renin 0·27 and 0·35 in Exp. 2. In Exp. 4, the corresponding figures were 0·26, 0·25, 0·22, 0·24. The ratio remained comparatively steady throughout each experiment, and was at its height before the renin response in four of the six experiments. Thus, although we may not legitimately infer from these experiments that renin

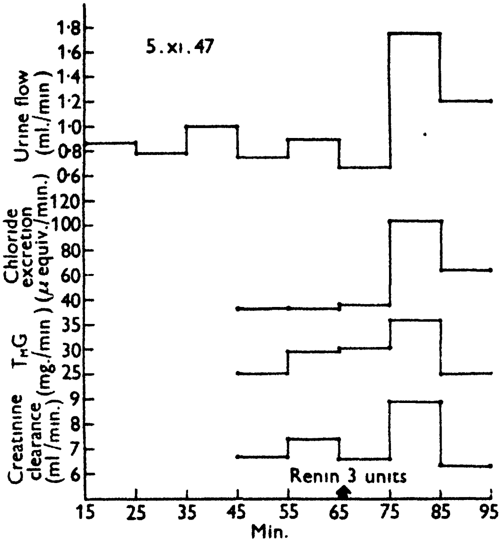

Fig. 8. Effect of renin on urine flow, chloride excretion, glucose Tm, and creatinine clearance in a rabbit anaesthetized with nembutal. Urine collections at 10 min. intervals.

has any definite effect on glucose Tm, we are justified in concluding that the striking increase in chloride excretion and change in the chloride content of the urine are not due to an increased flow of glomerular fluid down the individual nephrons. They are clearly due to a change in activity of the tubular cells.

DISCUSSION

The experiments here described show that hypertensin has an effect on the rabbit's kidney that is qualitatively identical with that of renin. In adequate doses both increase urinary output, both increase sodium and chloride excretion, both affect the inulin and diodone clearances in the same way, both produce a urine whose chloride content tends to approximate and slightly to exceed that of plasma. We have not been able to make a quantitative comparison between the actions of the two substances, because of the difficulty of securing a constant renal response to a given dose and because the effects of the two substances differ so much in duration. Nevertheless, our results, while offering no proof, are at least consistent with the hypothesis that the action of renin on the kidney, as on the vessels, is mediated by hypertensin.

It has been already shown (Pickering & Prinzmetal, 1940) that the diuresis produced by renin is not a simple consequence of increased glomerular filtration rate. This paper affirms that conclusion, and admits a similar statement for hypertensin. The changes in excretion of water, sodium and chloride are evidently of tubular origin. It now seems most improbable that a pressor diuresis is concerned. For urine flow and urinary chloride changes may be near their height when arterial pressure has regained the normal range (Pickering & Prinzmetal, 1940), and when the change in the conditions of glomerular filtration as revealed by the filtration fraction has subsided. Again, no evidence has been obtained of any increase in the volume of fluid presented by a functioning glomerulus to its corresponding tubule.

In the case of renin, evidence has been adduced that tubular reabsorption of water, of sodium and of chloride are diminished (Pickering & Prinzmetal, 1940). The observations recorded in this paper have revealed that hypertensin exerts a similar action. A measure of these changes may be found in Table 5 which shows the percentages of water, sodium and chloride entering the tubules that are finally excreted and the effects on these percentages of renin, hypertensin and hypertensin control. It will be seen that renin and hypertensin both diminish tubular reabsorption of water, chloride and sodium, while 'hypertensin control' is without demonstrable effect on water or sodium reabsorption. Moreover, the reabsorption of water, of sodium and of chloride is more or less equally suppressed by renin or hypertensin. These measurements, together with the demonstration that both renin and hypertensin produce a urine which approximates in chloride content to that of plasma, indicate that the chief effect of these substances is to suppress the differential reabsorption of water and chloride in the tubules.

In recent years much has been learned of the division of function within the nephron by the beautiful techniques developed by Richards and his school for obtaining fluid from the glomerular capsule and from different parts of the tubule. In both amphibian and mammal the glomerular filtrate may be accepted as an ultrafiltrate of plasma. Walker, Bott, Oliver & McDowell (1941) have shown that glucose is completely reabsorbed in the first half of the proximal tubule in rat and guinea-pig, and that in this part of the tubule water is absorbed at the rate of 12·5% of the fluid entering the tubules in rats and 7·5% in guinea-pigs per 10% of the total length of the proximal tubule. They were unable to collect fluid from the distal half of the proximal tubules, but, accepting a similar rate of water absorption, estimated that about 80% of the water presented to the tubules was absorbed in the proximal convolution. In amphibia, Walker, Hudson, Findley & Richards (1937) had found that the chloride content of the proximal tubule was higher than that of plasma, even when allowance had been made for protein differences, but had been unable to exclude a technical error. In the guinea-pig and rat the proximal tubule fluid was found consistently richer in chloride than plasma: 'the fluid plasma concentration ratio reaches an average of 1·40 in the first third of the proximal tubule and remains at this point without further increase throughout the second third' (Walker *et al.* 1941). The osmotic pressure of the proximal tubule was found to be identical with that of plasma, and Walker and his colleagues therefore suspected that the increased concentration of chloride was balanced by a fall in the concentration of some other ion. Beyond the proximal tubule little is known of division of function in the mammalian nephron. It has been inferred from comparative anatomy that the loop of Henle is the chief site of water absorption (Burgess, Harvey & Marshall, 1933), and that it is here in particular that the hormone of the posterior pituitary acts. Walker *et al.* (1941) measured in three rats the osmotic pressure of fluid obtained from the beginning of the distal tubule and found it actually significantly less than that of plasma. They suggested, therefore, that chloride absorption took place proximally to reabsorption of water, and possibly in the loop of Henle. Thus it would seem that in the diuretic phase the chief action of renin and hypertensin on the kidney of the rabbit, that of suppressing the differential reabsorption of water and salt, is located in the tubules distal to the proximal convolution. Although, as has previously been shown, the ratio urinary chloride to plasma chloride after renin tends to be greater than 1, the ratio has not yet been observed as high as 1·4, the value characteristic of proximal tubular urine in the mammal; it seems probable that the observed differences in chloride content of urine and plasma after renin can be accounted for by differences in protein content of the two fluids and the Donnan equilibrium.

It is by no means impossible that the tubular action of renin and hypertensin has a vascular basis. It is, for example, possible that blood is diverted from that part of the tubule concerned in differential water and chloride reabsorption, a possibility we have not been able to test experimentally. Peter (1909) and Huber (1909–10) have shown that there is a striking contrast between the nephrons whose glomeruli are situated in the periphery of the cortex and those originating in the juxta-medullary zone; the former have short loops of Henle, largely situated in the cortex, the latter long loops which plunge deeply into the medulla. It has been suggested that the urine formed by these two types of nephron is different (Trueta, Barclay, Daniel, Franklin & Prichard, 1947). The possibility arose that renin might divert blood from one type to the other. Following the injection of Berlin blue as previously described it was possible to demonstrate the interlobular arteries, their afferent glomerular branches and the glomerular tufts. We observed no consistent difference from the normal in the proportion of glomeruli filled nor in the distribution of these glomeruli in the kidneys obtained at the height of renin diuresis. We do not think, therefore, that the change in the quantity and quality of the urine secreted after renin can be due to diversion of blood from one type of nephron to another, but we cannot exclude a diversion of blood away from some part of the tubules.

We wish to thank Dr A. E. Barclay and Miss M. L. Prichard for advice about injecting the renal vessels.

SUMMARY

1. In the unanaesthetized rabbit, hypertensin increases the rate of urine flow, and conspicuously increases chloride excretion. Urinary chloride concentration, whether high or low initially, tends to approximate to that of plasma.

2. Adrenaline and tyramine, in doses giving similar rises of arterial pressure, act in a manner qualitatively similar to hypertensin, but with a much less conspicuous effect on urinary chloride.

3. In the doses used, hypertensin has no constant effect on inulin clearance, but always reduces diodone clearance and increases the filtration fraction. The effect of renin is similar.

4. In the anaesthetized rabbit renin has no conspicuous effect on glucose Tm during the period of diuresis.

5. The evidence is compatible with the view that the effect of renin on the kidney is mediated by hypertensin.

6. The results suggest that the changes in the volume and composition of the urine during the diuresis produced by renin and hypertensin are due to suppression of the tubular capacity differentially to reabsorb water, sodium and chloride.

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GRADED RESPONSES OF THE ISOLATED VENTRICLE OF THE TOAD FOLLOWING RELEASE OF ACETYLCHOLINE BY DIRECT STIMULATION OF VARYING FREQUENCY

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Experiments on the isolated heart of the guinea-pig (Spadolini & Domini, 1940) showed that, while acetylcholine in a concentration of 10^{-7} elicits the well-established inhibitory reaction on the heart, prolonged motor effects are caused at concentrations of about 10^{-9}. It was also shown that both inhibitory and excitatory effects are sensitive to atropine. This suggests that the action of acetylcholine occurs at the myocardial effector and excludes the possibility, supported by Hoffmann, Hoffmann, Middleton & Talesnik (1945), that the excitatory effect may be adrenergic and due to excitation by acetylcholine of nervous ganglionic elements or local chromaffine cells. These findings were first confirmed for other mammals by Laporta & Saviano (1943), and later by McDowall (1946), and aroused lively interest (Rodbard & Katz, 1947). Peruzzi (1948) has recently found that adequate concentrations of atropine are required to suppress the inhibitory and excitatory actions of acetylcholine on the heart. Later attempts by stimulation of the vagus nerve to produce the two effects obtained with acetylcholine have not hitherto given decisive results because excitatory effects from such stimulation are only obtained after administration of atropine. In these cases the possibility existed that an adrenergic mechanism might be responsible for the excitatory effect.

Observations by Bowditch (1871), Hofmann (1901) and Kruta (1937) showed that the mechanical response of the myocardium to rhythmic stimuli varied with the frequency of stimulation, and revealed the existence of an optimum frequency corresponding to maximal contraction. So far as I know no experiments have been made to investigate the possibility that the relation between frequency of excitation and size of myocardial response is due to release of acetylcholine. Bearing in mind Rushton's (1930) findings that electrical stimuli applied to muscular tissue excite first the efferent nerve endings therein, it was decided to study, with particular reference to frequency of stimulation, the effects of electrical stimuli, applied directly to the ventricle in order to ascertain whether vagal stimulation so caused can produce dual effects which are determined by the concentration reached by the released acetylcholine at the myocardial effector.

METHODS

The isolated ventricle of *Bufo vulgaris* was used attached to a Straub's cannula at constant level. Experiments were made in autumn when the heart of this animal appears to be particularly sensitive to acetylcholine. For perfusion oxygenated Ringer's solution for amphibians was used; for electrical stimulation Kronecker's inductorium with an electromagnetic rotating key arranged to utilize only 'make' shocks and to vary the frequency of rhythmic stimulation. Non-polarizable electrodes were applied to the base and to the tip of the heart.

RESULTS

Using slow faradic stimulation* of liminal or supra-liminal intensity the contractions of the ventricle were found to be all of the same height. In some cases, at the beginning of stimulation, the well-known staircase phenomenon was observed. Keeping the intensity unaltered and slightly increasing the frequency of faradic stimulation, it was found that at a certain frequency the ventricle responded during long periods of stimulation with a progressive increase in the height of the beats. When such experiments are repeated on an isolated ventricle beating with spontaneous rhythm, the effect of stimuli of a certain frequency is more marked (Fig. 1). The excitatory response steadily grows in intensity and involves both systole and diastole. Atropine added to the perfusion fluid in adequate concentration abolishes this excitatory response.

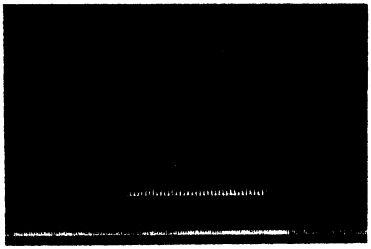

Fig. 1. Graded excitatory response of the isolated ventricle of *Bufo vulgaris* to faradic stimulation of adequate intensity and frequency. The ventricle was beating spontaneously.

If the frequency of stimulation is further increased the gradual augmentation of the ventricular contractions finally reaches a maximum, confirming the existence of an optimum frequency, as observed by Bowditch. The beats of the ventricle now follow the 'all-or-nothing' rule. If, at this point (Fig. 2), the frequency of stimulation is still further increased, the beats decrease gradually in height until they stop completely if stimulation continues. On the other hand, if stimulation is stopped and started again at a lower frequency, a gradual increase in the height of the beats again occurs.

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* It is not possible to indicate in these experiments any absolute frequency values since differences occur in the majority of cases. The effects described are not constantly met with in all instances; similar variation of response is shown by the toad's ventricle to acetylcholine or to vagus stimulation.

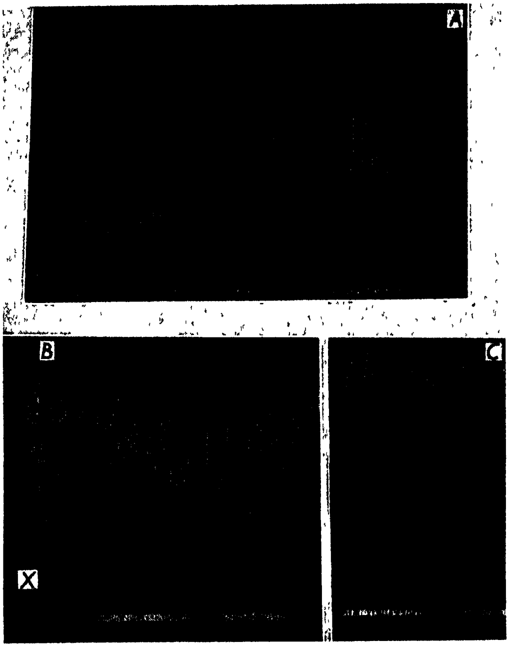

Fig. 2. Isolated ventricle: A, cessation of excitatory response to increased frequency of stimulation; B, development at higher frequency of inhibitory response with typical 'escape' phenomena; C, abolition at same frequency of inhibitory response by a small dose (10^{-4}) of atropine.

Atropine at a concentration of 10^{-4} (Fig. 2) changes a preceding inhibitory response to a marked excitatory response, although the high frequency of stimulation remains unchanged. This fact, together with (*a*) the immediate recurrence of vigorous contractions when the frequency of stimulation is lowered during the inhibitory response, and (*b*) the occasional escapes of ventricular beats during the inhibitory phase, suggests that the inhibition is an active phenomenon. The inhibitory response is not to be regarded as due to fatigue nor due, as Kruta (1937) surmised, to inability of the myocardium to follow high frequencies of stimulation.

That the inhibitory effect described is due to the release of an active principle is convincingly demonstrated by transference of the inhibition to another recipient heart attached to the donor ventricle by a double Straub's cannula.

The donor ventricle is electrically driven, and Fig. 3 shows that, during the gradual inhibitory response of the isolated ventricle to direct stimuli of suitable frequency, an active principle is released which exercises a very evident inhibitory action on a whole recipient heart. This action primarily affects the auricles which are known to be extremely sensitive to acetylcholine. When the auricles recommence to beat, the active principle released from the donor ventricle is still capable of evoking marked excitatory effects on the recipient ventricle. If the recipient heart is represented by an isolated ventricle beating with spontaneous rhythm, the most frequent effect is excitatory. Seldom does the donor ventricle release sufficient of the active principle to inhibit an isolated recipient ventricle.

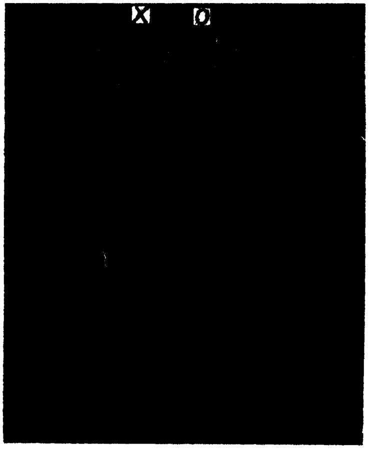

Fig. 3. Inhibitory response of donor ventricle with appropriate frequency of stimulation transferred to recipient heart. At *X* inhibitory effect of released acetylcholine develops first on the auricles. At *O* marked excitatory effect on the ventricle develops owing to dilution of transmitter.

Consideration of the dual action of acetylcholine (excitatory for minimal concentrations and inhibitory for higher concentrations) and of the disappearance of both effects when adequate doses of atropine have been administered allow the experimental results described to be attributed to the release of acetylcholine by vagal nerve endings. At low frequencies of stimulation the excitatory effects characteristic of small doses, and at higher frequencies the inhibitory effects characteristic of larger doses of acetylcholine, can be observed (Fig. 4). This dual action of acetylcholine provides an explanation of the two opposing factors, both varying with stimulus frequency, which Kruta (1937) postulated in explanation of his observations.

Also noteworthy is the action of atropine on cholinotropic cellular receptors. Atropine in concentrations of about 10^{-5} paralyses the inhibitory receptors and thus blocks the effect of larger doses of acetylcholine; atropine in concentrations of about 10^{-3} paralyses also the receptors that have a motor function and thus

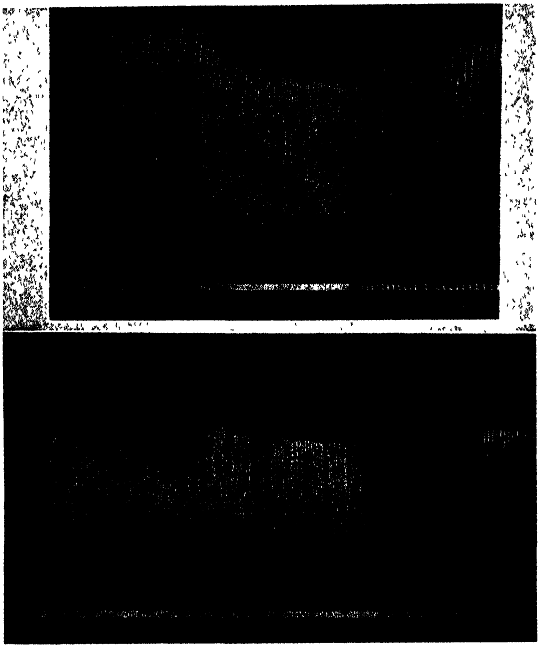

Fig. 4. Effect of successive changes of frequency of faradic stimulation on size of ventricular contractions.

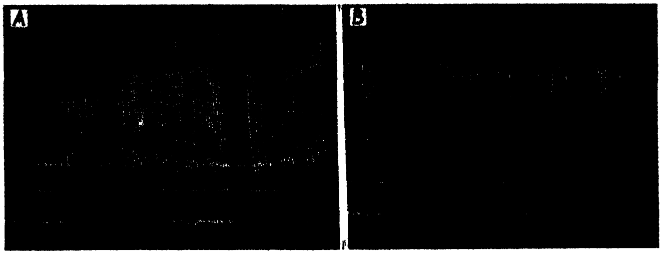

Fig. 5. A, Graded excitatory effect of adequate frequencies of stimulation of isolated ventricle driven electrically; B, abolition of excitatory effect by a stronger dose (10^{-3}) of atropine. Thereafter, whatever the frequency, ventricular contractions are unchanged in height.

blocks the excitatory responses characteristic of smaller doses of acetylcholine (Fig. 5). Evidently there are present cellular receptors *E* and *I* which have quite different affinities for atropine.

Whatever the frequency of stimulation, eserine always produces a gradual decrease in size of the ventricular contractions on account of its anticholinesterase action. The accumulation of the released acetylcholine results in the expected inhibitory effect characteristic of higher concentrations. Atropine in an eserinized preparation re-establishes ventricular contractions of remarkable height.

SUMMARY

1. By direct stimulation of the cardiac ventricle of *Bufo vulgaris* graded ventricular contractions can be obtained which increase or decrease in height according to the frequency of the faradic stimuli.

2. These effects, which are believed to be due to the excitation of intracardiac vagal nerve endings, can be transferred to another heart and seem related to the release of acetylcholine.

3. It is suggested that the effects are due to the dual action of acetylcholine on the myocardium according to the concentration reached at its site of action.

4. The gradual increase or decrease in height of the ventricular contractions observed in response to repetitive stimuli of appropriate frequency is suggested to be due to the accumulation of the acetylcholine released at each stimulus.

5. The experimental results explain the relation between frequency of stimulation and maximal height of ventricular beats previously noted by other authors.

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THE SITE OF EXCITATION IN THE NERVE TRUNK OF THE FROG

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When the sciatic nerve of a frog is stimulated through one pair of electrodes and the action potential is recorded at another pair, then upon reversing the direction of the stimulating current, the arrival time of the recorded impulse will be changed. This obviously is related to the fact that excitation occurs at the cathode, so when the stimulus is reversed and the cathode is shifted from one electrode site to the other, the conduction distance to the recording leads is changed. If this explanation is complete, when the electrodes are extremely close together, the latency change should correspond to the shift in excitation site only from one Ranvier node to the next, say 1 mm.

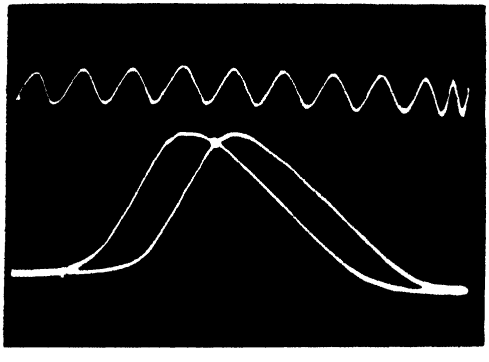

Fig. 1. Action potentials obtained by reversing the stimulating current through electrodes 1 mm. apart. Time scale: 5 kcyc. The latency change corresponds to 6 mm. difference in conduction distance.

Fig. 1 shows the result of reversing the stimulus applied through electrodes about 1 mm. apart. The two action potentials are seen to differ by about 0·2 msec. in their time of arrival, which corresponds (at the conduction velocity obtaining) not to 1 mm. but to 6 mm. shift in the site of excitation. Obviously there are two main explanations possible. (*a*) Despite the proximity of the electrodes, the sites of origin of the impulse are in fact separated by 6 mm. (*b*) The impulse starts from the manifest cathode in each case, but does not travel at the same speed. It will be observed that when the cathode is nearer the recording leads, the impulses must traverse nerve which has just been catelectrotonic. The recorded latency difference, therefore, might be the result of faster conduction through (recently) catelectrotonic nerve than through nerve (recently) anelectrotonic.

The present paper attempts to decide between these two possible explanations, in Part I by locating the site of the stimulus, in Part II by locating the site of origin of the action potential.

PART I. THE SITE OF THE STIMULUS

The possible explanations (*a*) and (*b*) above may be distinguished by an experiment which in principle is as follows.

The nerve is stimulated through a close pair of electrodes *X*, Fig. 2, with the anode towards the recording site. As seen in Fig. 1, if the current direction is reversed, the shock-spike interval will be reduced, so the electrodes may now be shifted to the left and a new position *Y* found where the increased conduction time exactly restores the shock-spike interval to its original value. Hence it will be possible to arrange two pairs of electrodes *X* and *Y* as shown such that each has the same shock-spike interval. So far this is a mere restatement of the prime observation, and does not discriminate between explanations (*a*) and (*b*). The distinguishing feature lies in the excitability of the nerve at various points.

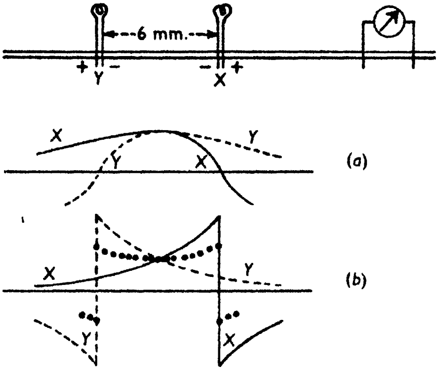

Fig. 2. Nerve excited by two electrode pairs adjusted to give the same shock-spike interval. Curves (*a*) and (*b*) indicate the excitability distribution to be expected if the excitation site is (*a*) 3 mm. extra polar, (*b*) exactly at the cathode.

According to explanation (*a*) the excitability is greatest not at the cathode, but at a point some 3 mm. away. So, if directly below the nerve in Fig. 2, a curve is represented showing the excitability of each point due to the stimulus

X alone, a relation such as the continuous curve XX, Fig. 2 (*a*), must be expected with a maximum 3 mm. to the left of the electrodes X. Similarly, the excitability due to Y alone will be given by the broken curve YY in the same figure. According to this explanation the maxima of the two curves have been adjusted to coincide, and that is why the shock-spike intervals are found equal. The critical consideration is as follows. Since X and Y each excite the same point on the nerve, their effects might be expected to be additive. In particular, if X alone is made threshold and then Y alone is made threshold, it should follow that $\frac{1}{2}(X+Y)$ should also be just threshold. For each stimulus would simultaneously contribute to the same optimum region of membrane half the current necessary just to excite it.

It would be quite otherwise with explanation (*b*). Here the excitability is maximal at the cathode and falls away with distance as shown by the curves XX and YY, Fig. 2 (*b*). But since in this case X and Y act at different and rather distant points, their simultaneous application will not be additive. For, if X and Y are each separately adjusted to threshold, then their average $\frac{1}{2}(X+Y)$, which is shown by the dotted curve of Fig. 2 (*b*), is seen hardly to exceed half-threshold at any point.

The question whether the locus of excitation is at the cathode or some 3 mm. away may be settled, therefore, simply by observing whether X and Y are additive in their effects as deduced from explanation (*a*), or nearly independent as deduced from (*b*).

METHOD

Trough. A modification of Lucas's fluid electrode was used to obtain small electrode separation with good symmetrical contact. The arrangement is shown in Fig. 3. The nerve was immersed in Ringer's fluid except for the peripheral end whence the action potential was recorded. AB and BC are silver boxes. The adjacent silver walls at B are cemented together separated by a thin layer of insulator, and a small hole is drilled through the middle, sufficient just to admit easily a frog's sciatic nerve. If the box AB is at one potential and BC at another, the change in potential of the nerve surface will be sharply localized in the region of B. So the electrodes X, Fig. 2, are realized by the hole in B, and the equivalent separation turns out to be about 1 mm. (see later Fig. 5).

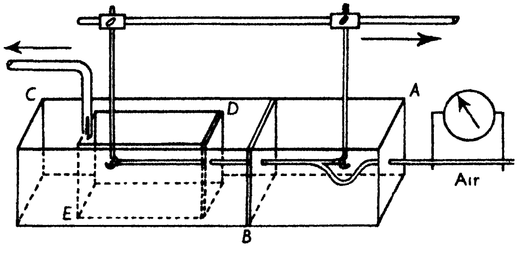

Fig. 3. Stimulating trough.

The electrodes Y, Fig. 2, result from a similar hole in the wall D of a third silver box DE. This is well insulated on the outside by paraffin wax, and the partition D is constructed, like B, of two separated silver plates, the outer of which is electrically connected to the box *BC*. A horizontal rack and pinion motion shifts the box *DE* so that the partition *D* slides over the threaded nerve. In this way the two partitions *B* and *D* may approach until their centres are 1 mm. apart. The setting is easily made correct to 0·1 mm. by means of a graduated circle on the pinion.

Preparation. The sciatic-peroneal preparation of the frog was used, dissected out in the usual way, monophasic records being led from the peroneal end, set up in moist air. The nerve was held by hooks applied to threads round the cut tibial branch and the largest of the cords of the sciatic plexus, the other cords being cut away to facilitate threading through the trough. These cuts were always far from the region stimulated. The hooks were mounted upon a second horizontal rack and pinion so that the nerve could be moved through the trough while leaving slack between the tibial hook and the recording leads. The locality chosen for excitation was the mid-thigh region which is fairly uniform.

Circuit. The box *AB* was kept at earth potential to reduce the stimulus artefact in the electric records. But it will be easier to appreciate the circuit if potentials are considered relative, not to earth, but to the potential of *BD*, since in this case the potentials of *AB* and *DE* will be proportional respectively to the stimulating currents *X* and *Y*, Fig. 2. The electrical circuit is seen in Fig. 4. A constant current pulse of a few tenths of a millisecond was passed through the primary

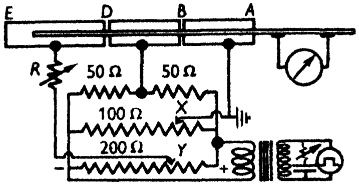

Fig. 4. Stimulating circuit.

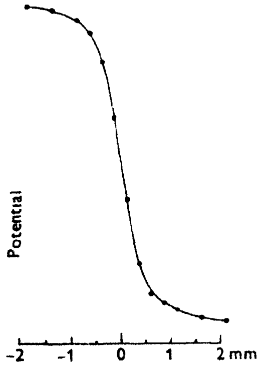

Fig. 5. Potential drop across slot *B*.

of a step-down transformer, capacity-shunted so that the wave in the secondary was more or less square. The stimulus at *B* and *D* was adjusted by the potentiometers *X* and *Y*. The object of this experiment is to apply stimuli *X* and *Y* in varying proportions and to plot one against the other for a constant physiological result. This plotting was conveniently performed by a simple device. The plotting paper was wound upon a cylinder whose axle was fixed by a bush to the knob of a radio-type potentiometer *Y*. The setting of *Y* is thus performed by turning the cylinder. The potentiometer *X* was of the straight tubular type, fixed parallel to the cylinder axis in such a way that a pen, spring-hinged to the slider, could be pressed on to the paper. Since the lateral displacement of the pen represents *X* and the vertical (i.e. rotational) displacement of the paper represents *Y*, it is clear that the pen point plots *X* against *Y* in the conventional manner. One is saved, therefore, the labour, time and error involved in reading scales, and transferring figures to graph paper.

Calibration. The linearity of the potentiometer readings as plotted was compared with the readings of a precision potentiometer. The divergence was always less than 0·5 mm. in the figures of this paper.

The independence of the two settings was not quite perfect, for the potential difference across slot *D* depended slightly upon the setting of *X*, and similarly across *B* it depended slightly upon *Y*. This was measured directly by two electric probes in the appropriate fluid regions connected by cathode followers to a differential amplifier (responding only $\frac{1}{1000}$ to symmetrical inputs). The interaction between X and Y is exactly expressed by drawing the axes of the graph slightly oblique as in Figs. 6 and 8. Thus a nearly vertical line, exactly parallel to the Y axis, represents various values of the potential across D with a fixed potential drop across B. In order that an equal drop across B and D should be represented by an equal distance along the two axes, a suitable adjustment was made to the resistance R.

Potential of the fluid. In order to find how the potential varied in the fluid along the nerve in the neighbourhood of slot B, Fig. 3, an enamelled wire bare only at the tip was threaded through in place of the nerve, the motion being controlled by rack and pinion. In this way the potential of the tip could be found as it passed through the hole in B, using a null-point method of potential measurement. The electric probe was connected to the grid of a cathode follower, and the output was neutralized by an accurately measured fraction of the 'stimulus'. Fig. 5 shows the potential plotted as a function of the distance along the 'nerve'. As the recording tip entered slot B, the potential changed rapidly, and the greater part of the change is completed within the space of 1 mm. The mid-point of the slot was taken to be the place where the potential change was exactly half completed, and the distance between the slots was taken as the distance between their mid-points.

RESULTS

It will be remembered, from the discussion on Fig. 2, that the experiment consists in two determinations:

(1) The electrodes Y (i.e. slot D) must be moved to such a distance from X that the shock-spike interval is the same for both.

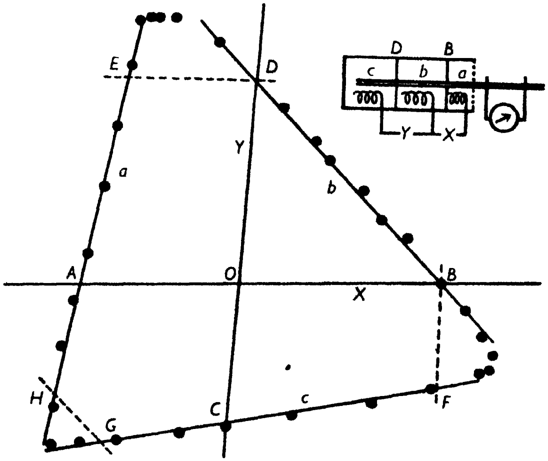

Fig. 6. Nerve excited by two slots B, D (inset) separated by 5·5 mm. At this separation the shock-spike interval was the same for excitation by X or by Y with cathode towards b (which is reckoned as the positive sign). The strength of X is plotted against Y for threshold excitation.

(2) The threshold for X alone and for Y alone is compared with the threshold for stimuli applied to X and Y simultaneously and in various proportions.

Fig. 7, frame (i) shows that condition (1) is approximately fulfilled when slot D is 5·5 mm. from B. The stimulus is a square pulse of 0·5 msec. duration starting at the downward 'escape' deflexion to the left of the record, and the action potentials are half maximal. The two superposed records are taken one from stimulus X only and the other from stimulus Y only and the two shock-spike intervals do very nearly coincide, as required by condition (1). If both X and Y are applied together in various proportions, the relation between them necessary for a half-maximal response is shown in Fig. 6, where Y is plotted against X in the usual way. The conditions of excitation are precisely as in Fig. 7, and indeed the two records of Fig. 7 are simply those from the points B and D on the curve of Fig. 6. For condition (2) of our experiment we are concerned only with the portion of Fig. 6 which lies in the first quadrant, i.e. the part between B and D. The expectations resulting from the alternative hypotheses (*a*) and (*b*) are as follows:

According to (*a*) the two stimuli X and Y both excite the same point of the nerve and hence they are simply additive. If, for example, X is 30% below threshold, then Y must be just 30% of threshold if the two combined are just to excite. Or, put graphically, if X is plotted against Y, the resulting threshold curve must be a straight line.

According to (*b*), on the other hand, the stimuli X and Y act at different and rather remote points (5·5 mm. apart in the present case) so that each stimulus would be fairly independent of the other. If they were quite independent, the curve between B and D would consist of two straight lines (the continuation of ED and FB) forming with the axes a 'square'. According to hypothesis (*b*), then, the experimental curve should approximate to this square.

It is clear, however, that the actual results of Fig. 6 fit the line BD rather exactly, and the 'square' not at all. The point B on the curve is the value when Y is zero and thus gives the X threshold for excitation at slot B (inset). Similarly, D gives the Y threshold for excitation at slot D. Intermediate points fall very close to the straight line BD. It may therefore be concluded that in the conditions of this experiment the site of excitation is about 3 mm. away from the manifest cathode, in the cathodal extrapolar region. Moreover, since Fig. 6 shows that the site of excitation is the same for points B and D on the curve, and Fig. 7, frame (i) shows that the shock-spike interval is the same for these two conditions of excitation, it follows that the conduction time is not appreciably influenced by passage through (recently) anelectrotonic or catelectrotonic regions of nerve.

The excitability curve

Before concluding Part I of this paper there are two further questions worth discussing. The first is this. In Fig. 6 only the portion of the curve between B and D has been considered. How is the rest of the curve to be explained? If it is assumed that the whole curve is a triangle, the interpretation is simple. The point B on the curve corresponds to stimulation by the slot B alone, which excites at the point b (Fig. 6, inset). Obviously the point A on the curve is just stimulation with this current reversed, which must excite at the point *a*. Thus the side *b* of the triangle represents stimulation at the nerve point *b*, and the side *a* presumably represents stimulation at the nerve point *a*. If point *a* were so far to the right that the stimulus *Y* could not directly affect it, then the side *a* of the triangle would be parallel to the *Y* axis. Actually it is somewhat inclined towards it, indicating that *Y* contributes about 10% of its value to the point *a*. Analogous considerations indicate that the third side of the triangle stimulates at *c* on the nerve.

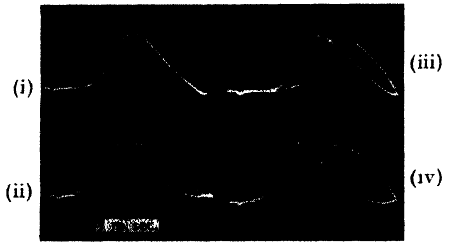

Fig. 7.

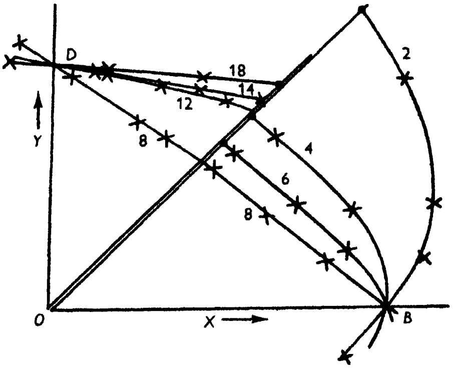

Fig. 8.

Fig. 7. Half maximal action potential records from the experiment of Fig. 6. Dotted reference line drawn perpendicular to base-line at 0·9 msec. after start of stimulus. Each frame gives superposition of two records from stimuli corresponding with the following points on the excitability triangle, Fig. 6: frame (i) *B* and *D*, frame (ii) *D* and *E*, frame (iii) *B* and *F*, frame (iv) *G* and *H*.

Fig. 8. Nerve excited by two slots as in Fig. 6. The curve relating *X* and *Y* at threshold is plotted for various slot separations indicated by the numbers (mm.). Only half the first quadrant of each curve is shown. Curve 8 in this nerve has the constant shock-spike interval.

This interpretation of the excitability triangle can be tested by measuring the shock-spike interval. For, since it has been seen that the velocity of conduction is not affected by the electrotonic changes involved, the latency of the response is simply due to conduction distance. It should therefore be expected that all stimuli lying upon one side of the excitability triangle should have the same shock-spike interval, but that this would jump suddenly to a new value on a different side of the triangle. This has been done in Fig. 7, where the vertical reference line has been drawn always 0·9 msec. after the beginning of the stimulus. Fig. 7, frame (i) shows that the latency is the same for *B* and *D*, and the impulse starts from the point *b*. Frame (ii) shows, superposed, the results of stimulating first at *D* (Fig. 6) and then at *E*. The record shows that *D* has again the latency of frame (i), and *E* has a latency about 0·2 msec. shorter, corresponding to a jump in the excitation point from *b* to *a*. Frame (iii) shows likewise an almost equal change of latency but in the reverse direction when excitation moves from *B* to *F* (Fig. 6), which corresponds to the increased conduction distance *b* to *c*. Stimuli at *G* and *H* should excite at *c* and *a* respectively, and hence the latency change in frame (iv) should be 0·4 msec. It is seen that this is approximately the case, and also that each curve in frame (iv) has about the same latency as the corresponding curve in (ii) and (iii). Thus frame (i) shows that there is no latency change along the side *BD*, and this last comparison of the remaining frames shows that there is no latency change along the other two sides. To a first approximation, therefore, the curve of Fig. 6 corresponds to excitation at these three different loci on the nerve.

Partition separation

The final question to be raised concerns a separation of the partitions *B* and *D* different from the critical value 5·5 mm. so far exclusively considered. According to the argument of this paper, the straight line *BD*, Fig. 6, is proof that *X* and *Y* at threshold each excite at the same locus *b*. If the slot *D* is shifted a little in either direction, this coincidence of loci will no longer occur. It is important therefore to be assured that the straight-line relationship obtains only for one critical value of electrode separation (namely where the two latencies are equal), otherwise the whole argument breaks down. This general assurance is given by Fig. 8, which is constructed from a series of complete excitability curves like Fig. 6, but each obtained with a different separation of slots *B* and *D*, as indicated (in mm.) by the number labelling each curve. If the nerve were uniform in every way, each curve would be symmetrical about the '45°' line through *O*. Also every curve would pass through the points *B* and *D*, because at these points one stimulus is zero, and hence it cannot matter how far or near it is. There is considerable variation from nerve to nerve in the value of the critical partition separation. In Fig. 6 it was 5·5 mm., the average value is perhaps 7 mm.; in Fig. 8 it was 8 mm., and the curve *BD* (8) is seen to be nearly straight. For compactness all the other curves have only one half plotted, since the omitted half was in fact more or less the reflexion in the '45°' line. Spontaneous changes in threshold during the determination of the whole set is compensated by scaling each curve to pass through *B* or *D*.

Turning to the results of Fig. 8, it is seen at once that the more the separation differs from the critical value 8 mm. either greater or less, the more the curves diverge from the straight line *BD*, and this always in the sense that threshold (=distance from *O*) becomes greater. This is precisely in accord with expectations, for since at 8 mm. separation the two excitation sites coincide, a movement of partition *D* in either direction will cause a separation of the sites and hence a less effective summation of the two stimuli. A detailed consideration of the shapes of the curve will not be undertaken here, but it will be noticed that it makes a good deal of difference whether the separation of loci results from the partition *D* being too close or too far. In the latter case (the upper set) each curve consists of a pair of nearly straight lines meeting (ideally) on the

'45°' line at an angle which approaches a right angle as the separation is increased. This is exactly to be expected, for as the electrodes become more separated, so Y receives a smaller and smaller fraction of X until at last it becomes quite independent, and the curve runs parallel to the X axis. Since in curve 8 the excitation sites coincide, in curve 14 they are situated 6 mm. apart, hence curve 14 shows the inclination to the X axis for a site separation of 6 mm. The side c of the triangle in Fig. 6 was interpreted as showing the same thing, since the distance between b and c was about 6 mm. The inclinations in the two figures are seen to be almost identical.

The curves for small-slot separation are much more complicated to interpret, and it will suffice here to make one observation concerning curve 2. Referring the description to the inset of Fig. 6, the site b will be 4 mm. from B (for the nerve of Fig. 8) and the distance BD 2 mm. for curve 2. Hence b lies on the anodal side of D and the effect of stimulus Y upon b is to *inhibit* excitation. That is why at the point B (Fig. 8), curve 2 is seen to run up to the right instead of up to the left. In curve 8, increase of Y involves a decrease in X, but in 2 increase of Y requires that X also be increased, i.e. Y inhibits.

The slope of each curve at B may thus be used to give information about the spread of excitability from slot D to the excitation site b. The method is developed in Part I of the next paper.

Enough has been said to indicate that the results of varying the position of the partition D are consistent with what went before.

PART II. LOCALIZATION BY DIRECT RECORDING

In Part I it was concluded that when the stimulating cathode lies very close to the anode, the impulse arises not at the cathode itself, but some 3 mm. distant on the cathodal side. This result was obtained by excitability measurements. It would be strengthened if the same could be found by recording the action potential directly at the origin of the impulse. It might perhaps be thought that it was impracticable to obtain any record from a nerve immersed in a large volume of saline, especially at the site of stimulation, but, by introducing electrodes within the connective tissue sheath (epineurium), quite large action potentials can be obtained. Their interpretation proved more difficult.

There has always been difficulty and sometimes confusion in the interpretation of experiments where one electrode was in contact with a nerve or muscle more or less immersed in fluid, and the other electrode in the fluid. This arrangement therefore was not attempted.

In the first experiments two fine insulated electrodes were inserted with their bare tips separated by 1 mm. along the axis of the nerve. The idea was to distinguish an impulse starting at the right of the tips from one to the left by noting the inversion of the recorded wave. This distinction is valid enough when the impulse runs uniformly past the electrodes in one direction or the other.

But when the impulse starts at the recording leads, both the electrodes become negative together (relative to a distant point), and that which is observed, namely the potential difference between them, is negligible. What appears in the record, therefore, is simply a long 'latency' (which in fact is simultaneous activity under both electrodes) followed by a wave of one sign or the other depending upon the direction in which the wave spreads faster. This obviously is a most misleading type of record for it strongly suggests that the nerve is quiet when in fact it is active, and that the impulse has arisen from a rather distant point when actually it starts at the recording leads. Something less paradoxical is needed.

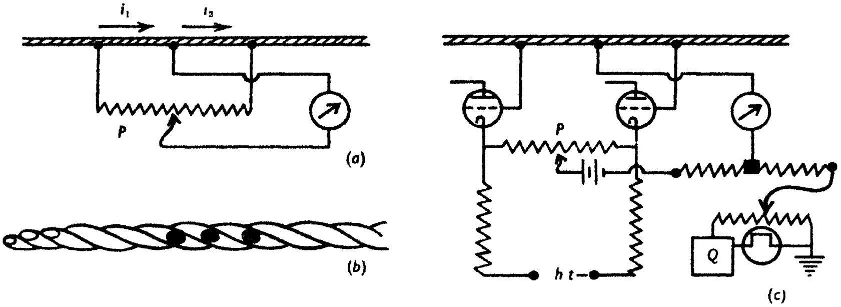

Fig. 9. (*a*) Diagrammatic circuit to measure (i_1–i_2). (*b*) Three fine insulated wires twisted together. The insulation is scraped bare on each wire at the black patch. Not at all drawn to scale. (*c*) Actual recording circuit to measure nerve membrane current. To avoid polarizing the bare areas (*b*), each lead goes to a valve grid and draws no current. *Q* is a suitable capacity network to balance out stimulus 'escape'.

Now the plainest property of a nerve element which indicates whether the nerve is passive, active in the element, or active in a neighbouring element, is the membrane current. This is the current which leaves or enters the nerve through its membrane (chiefly at the nodes of Ranvier). If the nerve is passive, this current is proportional to the stimulating current, and may be balanced out by a suitable capacitative network. In activity there is a local circuit where the current is inwards in the active elements and outwards in the neighbouring elements. The appearance, therefore, of an inward or an outward membrane current in records from a balanced network, signifies activity either in the recorded element or in the neighbouring region, respectively.

The membrane current may be measured by the tripolar recording arrangement shown schematically in Fig. 9(*a*). If current is passed down the nerve from an electrode far to the left of the figure to one far to the right, there will be no membrane current in the middle region where the recording electrodes lie, and (i_1–i_2) is zero. The potentiometer *P* is adjusted to give null deflexion in this case, and with this setting it is clear that, no matter what be the conditions of stimulation, the deflexion will always be proportional to (i_1–i_2).

Now this quantity is made up of two parts, the contribution from the stimulus, and the contribution from the action potential. The former may be eliminated from the records by using a subthreshold stimulus and feeding some fraction into a network so constructed that in the record $(i_1 - i_2)$ is exactly balanced out. The output is found to remain zero for all stimulus strengths below threshold. Above threshold the records show simply the membrane action current, and the critical question whether this is inwards or outwards is answered by noting whether the initial deflexion is up or down.

METHOD

The stimulating trough was the same as in Fig. 3, but as only one stimulation site was needed, the trough *DE* was removed. The recording leads were three fine enamelled silver wires No. 46 s.w.g. They were twisted together to form a 3-strand wire thread, and cut off obliquely to form a pointed tip (Fig. 9*b*). The whole was painted with shellac varnish, and the tip in particular was well insulated. The insulation was found to be satisfactory by testing between one strand and the next, and between the three strands together and the saline in which they were immersed. Now a small bright area was exposed by scraping away the insulation for about 0·2 mm. on the side of each wire. The three exposed areas were at intervals of 1 mm. along the axis of the wire thread and about 5 mm. from the insulated tip (see Fig. 9(*b*), which is not at all drawn to scale).

The frog's sciatic nerve was dissected out and set up upon a plane mirror so that, viewed through a binocular microscope (× 7), by reflected light, both the upper and the lower (reflected) surface could be simultaneously observed. The epineurium was pierced by a needle near the nerve junction at the knee, and the electrode inserted. The wire thread was always held in forceps lined with soft material to avoid damage to the insulation. The wires were carefully passed up the nerve within the sheath until the tip reached the cut branch to the hamstring muscles at the top of the thigh. It was fixed in this position by a ligature round the nerve with the contained wires near their point of entry. The nerve was then threaded into the stimulating trough with care to avoid much bending or relative movements between the nerve and the wires. Nevertheless, it is certain that considerable damage to the nerve fibres must have been done by this crude electrode insertion, and the action potential from the whole nerve distally was in fact rather small. The results to be described, however, were consistent and could be repeated for many hours. It was on account of the deformation of some fibres by the electrodes that the bright recording areas were placed some 5 mm. from the tip. For otherwise the region measured, instead of being uniform, would have been the junction between crushed and normal nerve, which might complicate the interpretation of the records.

Recording circuit. It is essential that no current should be drawn from the small bare areas of the recording electrodes, or they would become strongly polarized. They may not, therefore, be connected directly through the potentiometer as shown in Fig. 9*a*. The actual arrangement is shown in Fig. 9*c*. The middle electrode goes direct to the grid of the first amplifier valve (without grid leak) and the other two electrodes to cathode followers so that the potentiometer *P* may still act as in Fig. 9*a*, but without drawing electrode current. The output from *P*, suitably biased, is fed to the input of the amplifier, which may also receive a small fraction of the stimulating current either as a direct square wave, or as modified by the condenser circuit *Q*. The object was to balance out as perfectly as possible the 'stimulus artefact' and in the experiment of Fig. 10 all subthreshold records were nearly horizontal base-lines, except for the brief capacity spikes occurring at make and break of the square wave stimulus.

Procedure. (*a*) The amplifier was made truly differential by connecting all three electrodes together, varying their common potential relative to earth, and adjusting to zero any resulting output. (*b*) By a slight modification of the stimulating conditions a current pulse was passed uniformly in the fluid down the trough. The recording site was in the middle of this region, and the potentiometer *P* was adjusted to give no output. (*c*) The record now registers (i_1–i_2), and it is only necessary to ascertain the sign of the deflexion. This is found by leading a negative pulse into the fluid from a wire inserted near the middle recording lead. The downward deflexion which resulted provides the key for interpreting Fig. 10, for downward deflexion there must signify inward flowing membrane current, and hence membrane activity under the middle recording lead. (*d*) The stimulating trough was restored to its previous form, and action potentials recorded when the stimulating slot was in various positions relative to the nerve with its contained electrodes. Under the dissecting microscope the position of the wire tip could be directly observed through the epineurium, and the middle recording area was known to be 6·25 mm. from this. Thus the distance of the leads from the stimulating slot was exactly known. For each position the stimulating current was increased until just subthreshold. There was always some stimulus artefact, often a very large one, but this could always be exactly balanced out by suitable adjustment of the capacitative network *Q* (Fig. 9*c*).

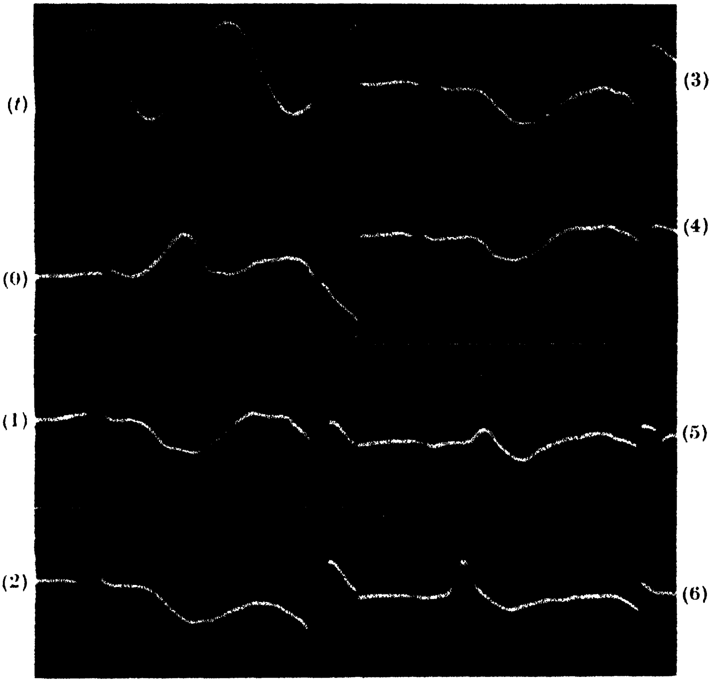

Fig. 10. Records of nerve membrane current obtained from the site of origin of the impulse when the nerve was immersed in a Ringer bath. Frame (*t*) 1 kcyc. Frames (0)–(6) records when the middle lead (Fig. 9*c*) was 0–6 mm. respectively from the centre of the stimulating slot. Downwards deflexion signifies inwards current, hence active nerve. The nerve becomes active simultaneously over the stretch 1–4 mm. (incl.) from the slot.

RESULTS

Fig. 10 shows a set of records taken at various distances from the stimulating partition. The stimulus is a square pulse of duration about 1·5 msec. The start and finish are accompanied by a capacitative spike, which does not seriously complicate the record. Apart from this all subthreshold responses were almost exactly horizontal lines at least for the first millisecond. The earliest detectable threshold responses were always in the same direction as the half-maximal responses of Fig. 10, though the latency was naturally longer because of the greater utilization time of the weaker stimulus. Strong stimuli, on the other hand, sometimes inverted a wave. This was doubtless due to the strong current spreading and exciting directly a region which formerly was only brought into activity by the propagated impulse.

Turning to the details of Fig. 10, frame (*t*) shows the time-scale of 1000 cycles. Frames (0)–(6) were obtained when the middle recording lead was 0, 1, 2, 3, 4, 5, and 6 mm. respectively from the centre of the stimulating slot. It is seen that when the distance is 1, 2, 3, 4 mm., the current crossing the nerve sheath starts downwards, i.e. in the inwards direction. So over this region the nerve becomes excitable at once, and the current flows out in the remoter regions seen at 0, 5, and 6 mm., and also beyond this, not here reproduced.

Conclusions. The results of this experiment stand in fair agreement with those of Part I. In view of the distortion due to electrode insertion some quantitative discrepancies are not surprising, and will not be discussed.

The main conclusion seems supported by both lines of investigation, for the impulse starts some 3 mm. from the manifest cathode in every case.

SUMMARY

1. It is found that when a nerve is excited through a pair of electrodes 1 mm. apart, the shock-spike interval depends upon the direction of the current. We should expect a change in conduction time due to 1 or 2 mm., but the change found corresponds to 6 or 8 mm. Is this due to the excitation site not being located at the manifest cathode, or to the conduction velocity not being the same in the two cases?

2. Two pairs of electrodes are arranged (Fig. 2) with opposite polarity to give the same shock-spike interval. If they really act both at the same place on the nerve, then the two stimuli will be additive in all proportions. If each acts at its manifest cathode the two will be more or less independent. They are found to be nearly exactly additive. Thus the locus of excitation is some 2 mm. or more from the manifest cathode, and conduction velocity is unaffected.

3. This conclusion is confirmed by recording the action potential at the excitation site. This was done in a nerve immersed in Ringer's fluid by passing the recording leads up within the epineurium. Excitation at threshold arose simultaneously over the whole stretch of nerve extending from the cathode for a distance of 3 mm.

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SPACE DISTRIBUTION OF EXCITABILITY IN THE FROG'S SCIATIC NERVE STIMULATED BY SLOT ELECTRODES

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The foregoing paper reached the conclusion that when a nerve is excited by slot electrodes, the impulse arises, not at the cathode, but 2 or 3 mm. away on the cathodal side. Fig. 2 (*a*) of that paper showed a curve representing the distribution of excitability about the electrodes which may be called the 'slot excitability curve', but the form was largely conjectural. The only point clearly established was that the maximum of the curve was some 3 mm. distant from the manifest cathode. The first object of this paper is to determine experimentally the whole slot excitability curve, and the second, to apply the curve so obtained to predict verifiable excitability results. In Parts I and II two different methods are described by which the slot excitability curve may be found, each strengthening some weak points in the other. In Part III the results are used to predict both the threshold and the latency of each point on the family of curves partly shown in Fig. 8 of the previous paper.

It is of course impossible to derive any of these conclusions from excitability measurements alone without assuming some guiding principle in their interpretation. The fact that Fig. 6 of the previous paper shows a linear relation at all seems to justify the assumption that excitability (=reciprocal threshold) at a point is an additive quantity. This assumption, which has been accepted by most workers in the past, forms the basis of the present analysis.

For the sake of concreteness the principal assumptions will now be set out in a form rather more definite and restricted than is necessary to justify the conclusions to be drawn from them. Their substantiation or modification will be postponed till the Discussion section at the end of the paper so that the bearing upon the results and conclusions may be appreciated.

Prime assumptions

1. The Superposition Theorem holds everywhere in the nerve structure. This theorem, which is a generalization of Ohm's Law, applies to all 'classical' circuits. In the present context it means that if a certain potential distribution

V_1 over the nerve surface produces a potential v_1 at some given point in the nerve structure (e.g. the membrane depolarization at some point), and if another distribution V_2 produces v_2 at the same point, then the distribution (V_1+V_2) over the surface will produce the sum (v_1+v_2) at the point.

2. A current pulse of fixed duration will excite a given point in a nerve if, and only if, the membrane depolarization at that point attains a critical threshold value. (This threshold usually varies somewhat from point to point along the nerve, and also changes with time, etc. The conditions are arranged to be as uniform as possible.)

It may now be stated more precisely what is meant in this paper by 'excitability' in a uniform nerve. Suppose v_c is the critical membrane depolarization required to excite any point—Assumption (2). Then if a given stimulus I produces a depolarization v_x at the point x, the local excitability at x is defined as v_x/v_c.

It follows from Assumption (1) that the same stimulus increased to $I(v_c/v_x)$ brings the depolarization at x exactly to v_c and therefore this point is just excited.

In practice one is concerned with threshold excitation of the whole nerve, i.e. threshold for that point which is most easily excited in the given conditions. Thus *the local excitability* at x is v_x/v_c and *the whole nerve excitability* is v_m/v_c, where m is the value of x where v_x is maximal.

PART I. SLOT EXCITABILITY CURVE

Determination by increments

Principle. It is required to find the excitability produced by a slot electrode at points on the nerve various distances away from it. What is measured is the excitability of the whole nerve, and the difficulty is to know exactly where the excitation is occurring, for unless one is sure of this point one cannot know what distance from the slot corresponds to the threshold reading.

This difficulty may be overcome by the arrangement of Fig. 1. If the nerve is excited at threshold by the slot X alone (Fig. 1a), with the cathode towards Y, the impulse will arise from a fixed point P, some 3 mm. to the left of X. Now if a very small stimulus is simultaneously applied through slot Y (say $1/n$ threshold where $1/n$ eventually becomes zero) it will not appreciably change the locus of excitation, but it will contribute to the excitability an amount which can be measured by observing what change is required in the strength of X, so that the excitation is once more exactly threshold. But the contribution which Y makes to the excitability at P depends directly upon the slot excitability curve of Y and in this way the curve may be determined. In fact, all that is necessary is to plot a little of the curve relating stimulus X to Y as in Fig. 8 of the previous paper, namely the portion of each curve near B. Then from the gradient of each curve at B the ordinate of the required slot excitability curve may be at once obtained simply by taking its reciprocal.

The truth of this follows readily from Fig. 1 (*b*). Let the curve XX be the slot excitability curve due to threshold current at slot X alone, and YY due to Y alone. Then YY will be simply XX laterally displaced by a distance equal to the slot separation. If stimulus Y is $1/n$ threshold, YY must be scaled down to $1/n$ the vertical dimensions (shown dotted). Now the contribution of this $1/n$ threshold stimulus ΔY is obtained by summing XX and the dotted curve YY/n. The maximum will still occur at the point P, but the ordinate will be decreased by y/n, where y is the ordinate of YY at the point P. Similarly, if the stimulus ΔY were reversed, the maximum would still be at P, but the excitability would be increased by the increment y/n. In our experiment, the threshold is kept constant by making a small change ΔX in X to neutralize the effect of ΔY. We have just seen that the magnitude of ΔX is y/n if $\Delta Y = 1/n$ hence

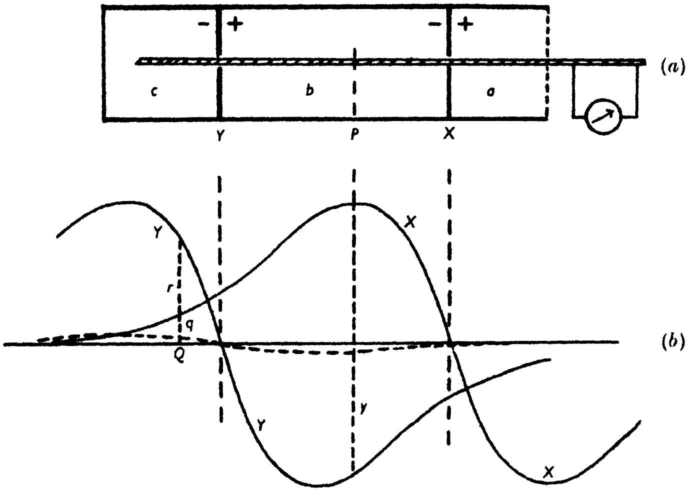

Fig. 1. (*a*) Arrangement for stimulating nerve by two slots X and Y. (*b*) Curve XX represents the space distribution of excitability due to slot X alone; YY due to slot Y.

$$\frac{\Delta Y}{\Delta X} = \frac{1}{y}.$$

In the previous paper, when discussing Fig. 8, the significance of the different slopes of the curves at B was pointed out. The present investigation uses this deliberately in order to determine exactly the slot excitability curve. For it now appears that the gradient of the experimental curve at B is the reciprocal ordinate of the slot excitability curve for abscissa distance YP. By a more rigid mathematical treatment it may be shown that the very small shift which occurs in the locus of the maximum does not affect this result, which is exact to the first order of infinitesimals.

Experiment. The frog's sciatic nerve was dissected out and set up in the manner described in Part I of the previous paper. The nerve was placed so that the fixed electrode X lay towards the peripheral end of the uniform thigh region, and the other electrode was moved to various points central to this. 'Threshold' excitation was either half maximal or some smaller fraction of the maximal action potential.

The relation between the stimuli X and ΔY was plotted directly on to paper by the recording cylinder described in the previous paper. In a preliminary physical calibration we determined the axes corresponding stimulus $X = 0$, and $Y = 0$, and also the line $X = Y$ which gives the relative scales on the two axes.

The actual experiment consisted in plotting the relation between stimulus X and Y for the region where the curve cuts the X axis to the right of the origin (i.e. when stimulus X has the polarity shown in Fig. 1). The experiment is repeated for various positions of the movable slot Y.

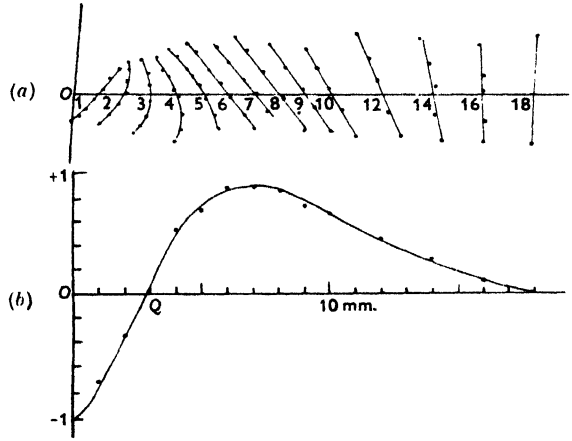

Fig. 2. (*a*) Each line plots the relation between the threshold value of stimulus through slot X (horizontal) and a small stimulus through slot Y (vertical), for slot separation indicated by the number (mm.) O is zero for Y but not for X. (*b*) Points derived from (*a*) as follows. O ordinate = minus the gradient where each curve cuts the horizontal. Abscissa = the number labelling the curve. This is the slot excitability curve with origin at Q.

RESULTS

Fig. 2(*a*) shows a set of curves cutting the horizontal axis for various slot separations indicated by the numbers (mm.). As determined they must of course all cut the axis at the same point, but in the figure they have been set clear by displacing each curve horizontally without changing its slope. Each is arranged to cut the X axis at a distance from O corresponding to its slot separation. As we view Fig. 2(*a*), moving from O to the right, we note that the slope of the curve where it cuts the axis changes from an inclination to the right, through vertical (at 3 mm.) to a maximum slope to the left at 6 or 7 mm., and then returns to the vertical (i.e. parallel to Y axis) at great distances. Plotting the reciprocal of these gradients (suitably corrected for axis obliquity and difference in scales), we obtain Fig. 2(*b*) where each point lies vertically below the intersection point of the corresponding curve in 2(*a*). If the nerve were uniform this curve should exhibit the following properties. It should cut the vertical axis at $y = -1$. It should have its maximum value $y = +1$. It should cut the horizontal axis at Q, half way between the maximum and minimum points, and the whole curve should be symmetrical about Q, which is the origin of the slot excitability curve.

These properties are only moderately fulfilled in our results. It is rather exacting to expect such symmetry in result from so asymmetric a stimulus and such accuracy in absolute values from measures of threshold increments. Yet we believe that the deviations of our results are due less to experimental error than to the non-uniformity of the nerve. For, repeating the experiment with the nerve unmoved, gives nearly the same curve, but shifting the nerve even a few millimetres, may displace the maximum of the curve by as much as 1 mm.

In the theory of this method it can be rigidly proved that when ΔX and ΔY are vanishingly small, then $\Delta X/\Delta Y$ is exactly the excitability at P (Fig. 1) due to the slot Y. Now the only reliable measurements of ΔX and ΔY are when they are not vanishingly small, but this introduces no great error in our result. For the curves of Fig. 2(*a*) run so smoothly that there is little ambiguity in drawing the tangent at the point of intersection with the horizontal, which gives the exact value required.

This method is tedious and it requires good precision in threshold measurement and stability of nerve since we base the results upon a 5% change in threshold. But despite this we consider it our most reliable method of determining the slot excitability curve.

PART II. SLOT EXCITABILITY CURVE

Determination by symmetry

The method now to be described overcomes some of the disadvantages of that in Part I, for it is easy and quick to perform and the slot excitability is measured directly, but there are other defects, as will appear. The nerve is stimulated by the two slots X and Y, Fig. 1 (*a*), but arranged symmetrically so that the region b is at some negative potential while a and c are both earthed. The threshold is obtained for various slot separations. From this relation it is theoretically possible to obtain the whole slot excitability curve in the case of uniform nerve, but in practice the nerve is not uniform enough, and the curve is reliable only up to the maximum and a little way beyond.

Perhaps the clearest and most interesting way to present this investigation will be to predict the results from the curve of Fig. 2. First then we shall show how this prediction may be made; then we shall describe the experimental determination, and check prediction against observation. Finally, by reversing the argument, we shall deduce the slot excitability curve from these experimental results.

Prediction. Our problem is this. If we know the slot excitability curve and assume the nerve uniform, what will be the threshold for symmetrical stimulation with any slot separation and at which point on the nerve will excitation arise? In principle a method of solution is obvious enough. We may draw the slot excitability curve in two positions, one for each slot. The summed ordinates give the resulting excitability for each point on the nerve. Excitation will occur at the point of maximum excitability and threshold will be the reciprocal of this maximum ordinate. But if a new curve must be plotted for each slot separation, the process will be rather tedious. The following method is quicker and more illuminating:

The slot excitability curve is given by OAB, Fig. 3. Trace this curve on a piece of card and cut it out together with a portion of the axis OX. Turn the card in its own plane through exactly 180° so that O_1X_1 is again horizontal and place it to touch the curve OAB at (say) P. Then it will be shown that the co-ordinates of O_1 give slot separation and the corresponding whole nerve excitability, while the co-ordinates of P give the distance from the fixed electrode at which excitation arises, and the contribution of this electrode towards the total excitability. Clearly, by sliding the card round the curve OAB, the required information is instantly given for every value of the slot separation.

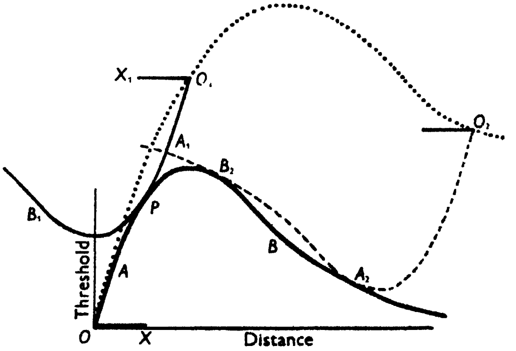

Fig. 3. Construction for predicting the results of symmetrical stimulation with two slots, when the slot excitability curve is given (see text).

The truth of the above interpretation of the figure will be evident upon examination, for the meaning of the curve OAB is that the ordinate represents the excitability at each point due to the fixed slot at O. Clearly a second slot at a distance from O equal to the abscissa of O_1 will produce the excitability shown upside down by the curve $O_1A_1B_1$. At the point P the sum of the excitabilities due to the two slots is equal to the ordinate of O_1. At every other point the sum of the two excitabilities is less than this by an amount equal to the gap between the two curves. Consequently, P is the point excited at threshold and the whole nerve excitability is the ordinate of O_1.

This establishes all the features of the foregoing interpretation without any reference to the fact that OAB and $O_1A_1B_1$ are the same curve. The construction is thus seen to be applicable also in the more complicated cases of asymmetrical slots, with which we are not here concerned.

Let us now consider the way in which the card slides over the curve OAB. So long as P lies near the maximum of OAB the curves will be in contact at one point only, and this from symmetry must be the mid-point of the line OO_1. As the curve moves far to the right a new state of affairs arises as shown by the interrupted curve $O_2A_2B_2$. The mid-point between the electrodes O and O_2 is here less excitable than regions A_2, B_2 on either side of it, and excitation will arise from one or other of the two contact points, which are seen to lie near the maximum point of each curve.

It is important to know over what range there is only one contact point, and where there are two. This is best found empirically by sliding round the card. It turns out that there is only one contact point P as this slides round from O up to the maximum and for some way down the right side of the curve. But at the point of inflexion (where the curve changes from convex upwards to concave) the contact point becomes double. It is conceivable that the contact point might become double before reaching the inflexion point, by the tail of each curve hitting the summit of the other, but in practice the curves are not quite the shape to do this.

The answer to our problem then is this. The required excitability curve is given by the (dotted) path traced by O_1. This is exactly a reproduction of curve OAB (at magnification $\times 2$) so long as there is a single contact point, and the site of excitation at threshold will, in this range, be half way between the electrodes. But as soon as the inflexion point is reached there are two sites possible for excitation and these rapidly separate to remain close to the maximum point for each slot electrode. From now on, the excitability will be nearly the sum of the maximum of one curve and the tail of the other. We turn to the experiment.

Apparatus. This was the same as in Part I. The potentials of regions a and c (Fig. 1) were made the same by earthing both the corresponding silver boxes, and it was verified that electric probes in the two solutions then registered no potential difference. Thresholds were measured by a potentiometer calibrated against a precision instrument. The only new feature was the measurement of the shock-spike interval. This turned out to be surprisingly accurate and rapid and was done as follows:

A small glass rod had one side ground rough, and it was then drawn out so that it tapered down to 0·2 mm. in a total length of 10 cm. The roughness was not obliterated by this process so that when a light was shone into the wide end of the rod, some was emitted all the way down instead of being entirely confined. Viewed from the side, therefore, the rod illumined by a 2 V. flash-lamp appeared as a fine yellow luminous line. It was mounted vertically just in front of the cathode-ray tube (whose time-base was horizontal) in such a way that the line could be set in any desired position across the face of the tube and the setting read upon a scale illumined by the same lamp (screened from the front). The green double beam tube was set so that the amplified action potential was applied equally to both deflectors. This resulted in the two beams giving mirror-image records which were arranged so that the monophasic waves approached each other. If the two base-lines were separated by a distance equal to the excursion of the maximum action potential, clearly the half maximal action potential is given by the conditions where the two reflected waves just touch. This is a very good index to observe, since the beam becomes twice as bright when the two thin lines overlap. Often 0·2 maximum action potential (or some other small value) was taken, by observing the coincidence of the mirror peaks when the base-lines were separated by some fraction of the maximal excursion.

The vertical yellow line could be set to an accuracy of 1 mm. upon the coincident green peaks of each 'threshold' setting, and this at a sweep velocity of some 25 mm./msec. means that the point of excitation on the nerve could also be localized correct to 1 mm. It is doubtful, however, whether all the processes contributing to the shock-spike interval remained constant to 0·04 msec. and the inferred excitation sites often exhibited an apparent fluctuation of 1 mm. or so.

Experiment. The nerve was dissected out and set up as before. As far as possible the most uniform stretch of nerve was selected for the site of excitation. This was found electrically by determining the threshold with a slot separation of 1 mm., as the nerve was gradually drawn through the slots by the rack and pinion mechanism. The most uniform region turned out to be the branch-free stretch in the thigh; other parts unfortunately had a *lower* threshold and therefore stole the site of excitation when slot separations were large.

The experiment was usually performed as follows. At an electrode separation of 1 mm. the threshold and latency were determined. Then the slot separation was increased by 1 mm. and at the same time the nerve shifted through the slots 0·5 mm. so that the same point on the nerve always lay midway between the electrodes. Thresholds and latency were again measured. In this way the double relation was obtained for a wide range of separations. Readings were now repeated in reverse sequence. If the two sets agreed closely the average was taken, otherwise they were rejected.

In order to be able to convert the observed latency differences into shift of excitation site, conduction velocity of the nerve was found, using only one slot and obtaining the linear relation between its setting and the resulting latency. In some experiments (e.g. Fig. 5) the nerve remained fixed, so the expected excitation site shifted along the nerve.

RESULTS

Fig. 4 shows the effect of performing this experiment upon the nerve which was used in obtaining Fig. 2. The upper set of points, L, represents the latencies found when the stimulus was half-maximal at slot separations shown by the horizontal scale. Latencies are converted into distance of propagation as shown by the scale on the left whose zero is fitted to the left-hand points. The middle set of points, A, represents the excitability measurements in this experiment. The lower points, B, are those of Fig. 2 but plotted with origin at Q and scaled so that the maximum height is just half the maximum of A.

The curve of B is drawn freehand to fit the points as in Fig. 2. The curve A is constructed from B according to the method of Fig. 3. It is exactly the same as B but at magnification × 2 for a distance 7 × 2 mm. and thereafter lies above it. The construction of Fig. 3 also predicts the excitation site, from the contact point P. As we have seen, so long as contact is single, the excitation point will be midway between the slots. In our experiment the nerve was always adjusted so that this corresponded to the same point on the nerve. The predicted latency variation is therefore zero over this range, as shown by the curve L. Beyond the point of inflexion, however, two contact points appear and rapidly diverge, so alternative sites are possible as shown by the two branches of L.

Now, comparing prediction with observation, we see that up to 10 mm. separation the excitability is exact, and the latency shows only two points of deviation. For greater distances the excitability is always too high and the latency mostly too long even for the more delayed branch of *L*. This is consistent with the initial observation that the nerve in the region of the cut branch to the hamstrings has a decidedly lower threshold than in the more uniform mid-thigh region. As the slot *Y* approaches this excitable region, therefore, the excitability will appear higher than predicted, and the latency greater.

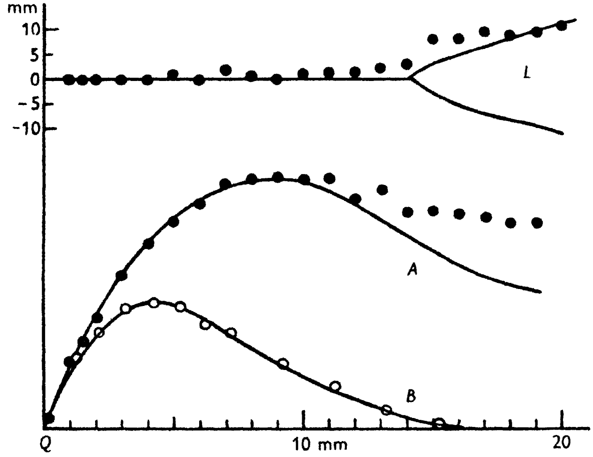

Fig. 4. Curve *B* with points is a replot of Fig. 2(*b*). Curves *A* and *L* are predicted from *B* by the method of Fig. 3. Points *A* and *L* are respectively the observed excitabilities and latencies in symmetrical stimulation. Latency is converted into excitation site by the vertical scale (mm.).

A similar set of determinations upon another nerve is given in Fig. 5(*a*). For present considerations the continuous line is to be neglected. The dots are obtained by the method of increments as in Fig. 2, the crosses by symmetrical stimulation as in *A*, Fig. 4, but plotted at half scale. It is seen that here again the coincidence is good up to, and somewhat past, the maximum.

In this experiment the nerve remained still, hence the predicted latency curve *L* will appear plotted upon a slope. The observations are in two sets, left to right and then right to left. They fit the predictions a little better than in Fig. 4.

Determination of the slot excitability curve

By the construction of Fig. 3 we have shown that in uniform nerve, the excitability curve for symmetrical stimulation is necessarily the same as the slot excitability curve (at magnification × 2) up to some way past the maximum point. If we may argue conversely, the required slot excitability curve may be obtained in this range simply by plotting the results of symmetrical stimulation at magnification $\times \frac{1}{2}$. We have seen how well this method agrees with that of Part I in the nerves of Figs. 4 and 5(*a*) and this agreement is the more impressive when we note that the determinations of Fig. 4 both have their maximum at 4·5 mm. and differ very much from the two sets of points in Fig. 5 whose maximum lies at 2·7 mm.

It thus appears that the slot excitability may be found rapidly and accurately by the method of symmetrical stimulation, but that this is reliable only up to the maximum of the curve or so far beyond as shows no latency shift.

PART III. THE GENERAL CASE OF STIMULATION BY TWO SLOTS

According to the prime assumptions of this paper, local excitability is an additive quantity, so it follows that the effect of two or more slots may always be computed simply by summing the ordinates of the corresponding slot excitability curves, suitably displaced and scaled. In uniform nerve, the excitation will arise from the point of maximum total ordinate and the threshold will be the reciprocal of the maximum ordinate height. We have in Part II already treated the case of two symmetrical slots and seen good agreement between prediction and experiment. In the present section we apply different currents through the two slots and for any given strength Y, find the value of X which just brings the stimulus to threshold; we also measure the latency (which indicates the excitation site). This is repeated for different values of the slot separation. This experiment was indicated in Fig. 8 of the previous paper, where a portion of each curve is plotted, but there the question was simply whether the curves were straight at more than one critical slot separation. In the present section the curves are plotted more fully (Fig. 5) and a comparison is made with theoretical predictions.

Experiment. This was carried out exactly as in Part I of this paper, except that instead of plotting the curve only where it cut the positive X axis, the complete curve was obtained. At the same time the latency of response was observed by the method described in Part II, for each point on the curve.

The chief difficulty was to get a uniform stretch of nerve, and when the excitability curve was not roughly symmetrical about the 45° line through O, it was rejected. By careful handling and selecting of a suitable mid-thigh region it was usually possible to get good curves when the slots were separated by only a few millimetres, but it is not easy to obtain a uniform threshold for excitation from every point in a nerve stretch of 12 mm. extent, and this is already required for a slot separation of only 5 mm. So larger separations are usually rather asymmetrical.

RESULTS

These are shown in Fig. 5(*b*)–(*f*). The experimental points were nearly symmetrical about the 45° line and only the upper half of each curve is plotted. The lower half is occupied by the theoretical curve derived from the slot excitability curve determined upon the same nerve. This is shown in Fig. 5(*a*) and has been already discussed in Part II. In each of the upper-half curves (*b*–*f*) the circles represent the experimental points and show the relation between the stimuli X and Y. The numbers are derived from latency measurements as follows. From the measured conduction velocity the observed latency is converted into propagation distance. Now we have seen (Part II) that in symmetrical stimulation, the excitation site is usually midway between slots. This is the condition where the curves *b*, *c*, *d*, *e* cut the 45° line. All other points are found to have a propagation distance less than this and the difference (shown in millimetres by the numbers on the curve) is therefore the distance of the excitation site from the mid-point between the slots.

Calculation. In principle this is easy enough, as we have seen, but the work of scaling and summing two curves for every point to be determined is very heavy. We shall therefore introduce a method less tedious to perform and more instructive to use. In the previous paper it was argued that if the relation between X and Y was linear, then the excitation site was at a fixed point on the nerve. Here we argue the converse, namely that for any fixed point on the nerve to be just excited requires that the stimuli X and Y should lie on a determined straight line. This follows at once from Fig. 1 (*b*) where the curves XX, YY represent the slot excitability curves due to unit current at the two slots. Consider the excitability at some point Q, and let q, r be the ordinates here of the curves XX, YY. It is clear that unit current at slot X produces an excitability q, hence a current x will produce excitability qx. The excitability produced at Q by currents x and y in the two slots will thus be $(qx+ry)$, and if this excitability is to remain threshold at Q we have the linear relation

$$qx+ry=\text{constant}.$$

If the nerve is uniform, the constant is the same for all points. It simply alters the magnification of the figure and may be made unity. Hence the condition for excitation at any point Q is exactly determined by the two ordinates at Q, namely q and r (Fig. 1). The result is that X plotted against Y is the straight line which cuts the axes X, Y at the points $1/q$, $1/r$ respectively.

It is clear then that if we know the slot excitability curve, we may plot it twice, displaced as in Fig. 1 according to the electrode separation, and for any point Q chosen, draw upon the XY excitability diagram, the line

$$qx+ry=1$$

which corresponds to threshold excitation at Q. The distance of Q from the point midway between the slots is known and hence the line may be labelled with this number of millimetres, thereby defining the excitation site.

In Fig. 5 the slot excitability is shown in (*a*), and the lower half of the other curves are determined from it by this method. Now the threshold for the whole nerve is always given by that portion of line which is first encountered when travelling straight from O in any direction. The threshold curve experimentally obtained should therefore be the *envelope* of the family of lines. Of the

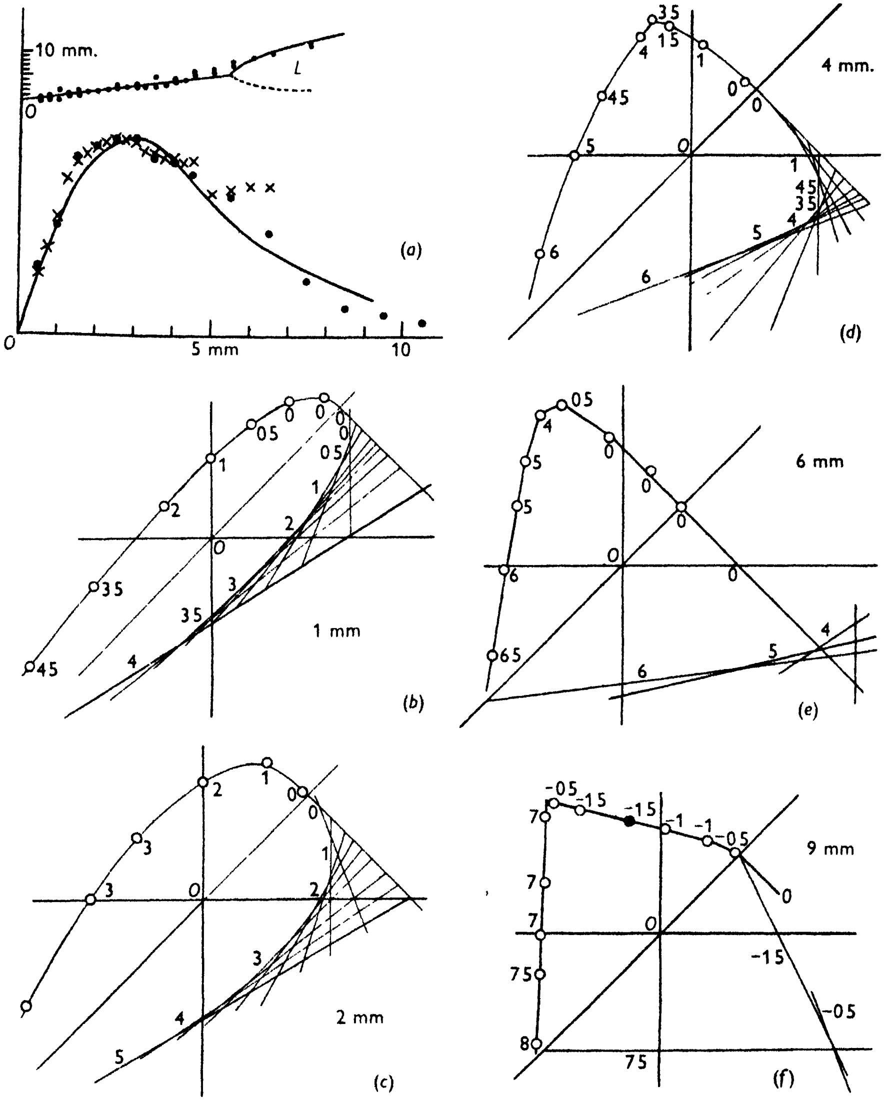

Fig. 5. (*a*) Slot excitability curve determined by increments (dots) and by symmetry (crosses). Curve *L*, expected latency, together with observed points. (*b*)–(*f*) Results of stimulating with two slots *X* and *Y* separated by 1, 2, 4, 6, 9 mm. respectively. In each figure current through *X* is plotted against *Y* for threshold. Each threshold point has a number indicating the response latency. This has been converted into excitation site measured from the point midway between the slots. The experimental results were symmetrical about the 45° line and only the upper half is shown. Below the line are the results deduced from the slot excitability curve drawn in (*a*). The envelope and numbers should be a reflexion in the 45° line of the observed results.

various kinds of non-uniformity in the nerve which invalidate our results, the most serious is the variation in threshold from point to point. This is a variation in the constant of the linear equation. The method of envelopes shows at once how this will affect the curve, for if a given point has an abnormally high threshold the direction of the corresponding line will be unchanged, but the line will recede from O, and will soon be masked by the superior excitability of the neighbours. For this reason, in comparing calculation with observation, rather more weight should be placed on the slope of the curve at a given number, than upon its distance from O.

Comparison between theory and observation

The actual curve used to obtain all the straight lines in Fig. 5 is the continuous line in Fig. 5 (*a*). This line was chosen rather than the curve exactly through the points, because it gave a distinctly better fit to the curves (*b*)–(*f*) and did not deviate from the experimental points more than usually happens when the region of the nerve investigated shifts a little, though it must be admitted that the two methods of obtaining the experimental points do not show so much deviation. It is clear that the theoretical curves for 1, 2 and 4 mm. are in close agreement with observation both as to threshold and excitation site. The curve for 6 mm. corresponds to Fig. 6 of the previous paper. The theoretical curve is almost exactly a triangle, each side corresponding to a region of constant latency. The experimental points correspond well enough, but the region at 4 mm. from the mid-point appears to have a raised excitability, so line 4 is too near the origin, and the corner of the triangle somewhat rounded off.

In (*f*), the curve for 9 mm., the excitation site is measured as usual from the point midway between the slots, though this point is never itself excited at threshold. The black point is given the value $-1{\cdot}5$ mm., and all other points have the site calculated in the usual way relative to this. It is seen that observation and prediction agree fairly well. In addition to these five curves we have obtained two others entirely comparable. At 3 mm. the curve is intermediate in form between 2 and 4; at 12 mm. the two slots are practically independent of each other and the results form a square.

In observing the rather good fit of observation with theory over a whole family of curves, the question which comes to mind is 'How far is this fit due to ingenious mathematical manipulation and the careful selection of results?' In Fig. 5 we have in fact improved the results somewhat by operating upon the continuous curve 5 (*a*) which does not lie precisely through the points. The grounds for doing so were stated above. But if a smooth curve through the points were taken instead, the results would still fit the observations rather well. The slot excitability curve being given, there is no latitude whatever in the mathematical manipulation. Each family of lines, and hence their envelope, is rigidly determined by the construction described above; only the scale of plotting is free, and that has been chosen so that theory and observation meet on the 45° line.

Observation will not agree with expectation nearly so well as in Fig. 5 unless the stretch of nerve selected for the experiment is very uniform. The test for uniformity we have used is the symmetry of the complete experimental curve about the 45° line. If, as commonly happens, the two halves are not approximately images, the nerve cannot be uniform, and the results were rejected. Otherwise they were always accepted, and always fitted the theory pretty well. In only one other preparation did we perform the complete set of determinations as in Fig. 5. It was upon the nerve of Fig. 4. In this case the agreement between theory and observation was slightly better than in Fig. 5.

DISCUSSION

If the prime assumptions set out at the beginning of this paper are accepted, the sequel is consistent enough. But without qualification they cannot be granted.

Assumption 1. *The Superposition Theorem holds throughout the nerve structure.*

This theorem (or deductions involving it) has been assumed in the great majority of nerve theories. But experimental evidence has been accumulating and now appears conclusive that it is not true of nerve around threshold conditions. Physical measurements (Cole & Baker, 1941) by alternating currents have shown that the membrane resistance diminishes at the cathode of a subthreshold stimulus. Measurements of the current-voltage relation at the membrane (Cole & Curtis, 1941; Hodgkin, 1947) indicate a fairly complicated situation. In addition to the a.c. resistance change there is the characteristic activity of the membrane in generating a potential which may culminate in the fully propagated wave. These measurements have been made upon single non-medullated fibres, and with medullated nerves the case is not so clear. There may well be a large inert resistance in series with the membrane. However, Katz (1947) has recorded subthreshold action potentials in a frog's sciatic trunk, and this has been studied in greater detail in single medullated fibres by Stämpfli (1946).

It has been known, moreover, for many years that the Superposition Theorem does not account for the effect of applying two stimuli with the same spatial configuration, but a different time course. When a test shock is used to measure the efficacy of a square wave (Erlanger & Blair, 1931; Rushton, 1932) or a previous shock (Katz, 1937), an increase in excitability is found near threshold beyond that expected from superposition. This can be explained as the result of local membrane activity.

Returning now to our Assumption 1, it is clear that it cannot be universally true, but its application in this paper has been undoubtedly successful. We need to be satisfied why it is legitimate to superpose stimuli with the same time course but a different space configuration, but not the other way round where the electrodes are the same but the time course is varied. The explanation is probably as follows.

The Superposition Theorem holds well enough for anodal currents, and weak cathodal currents; it is only in the neighbourhood of actual excitation that it breaks down. Now in all the excitability measurements of this paper we are simply concerned with threshold excitability at some point due to a stimulus of fixed duration, and this requires that the current leaving the nerve axis cylinder at the point should have a fixed value (see Assumption 2, below). A fixed current at the excitation site is thus secured in every measurement; what is varied is the distribution of the current in the remoter regions. But there the current has the density and polarity which satisfies the Superposition Theorem. Assumption 1 is valid, therefore, in these experiments because they secure that in the only region where the Superposition Theorem is not valid, the current is invariant.

Though the above explanation may well be correct we are not convinced that it necessarily is so. In Katz's results (1937) superposition begins to break down when the local excitability exceeds 50%, and in our experiments the space distribution about the cathode changes somewhat as the conditions of stimulation are varied. In these circumstances we cannot estimate how far the cathodal conditions may be regarded as invariant, but at present it seems reasonable to accept Assumption 1 as applicable to the conditions of our experiment.

Assumption 2. The necessary and sufficient condition for nerve excitation is that the stimulating current leaving the sheath at some point should attain a fixed value.

Nearly all the excitation theories since Nernst (1899) have assumed that a critical depolarization at some point of the membrane was the necessary and sufficient condition for excitation. But this is not true for non-medullated nerve, for Hodgkin (1938) showed that the transition from local to propagated response depended in general, not only upon the strength of stimulating current, but also upon the spatial spread of excitability from the cathode. In the case of medullated nerve, on the other hand, Tasaki (1939) gives evidence which goes some way to prove that a critical depolarization at a single node *is* the condition sufficient for propagation. Using tripolar stimulation with electrodes applied to three adjacent nodes of a single fibre, he obtains results which fall exactly upon a triangle (his Fig. 2). This seems to indicate that the same current is required to excite a single node no matter what (subthreshold) current is applied to the neighbouring nodes. And he draws attention to the fact that his results show excitability to be a strictly additive quantity.

In our paper the conditions of stimulation are far less critical than in either Hodgkin's or Tasaki's. The slot excitability has a gently rounded maximum, and so in all our experiments the impulse might be expected to arise almost simultaneously over several millimetres of nerve. This is what in fact was found to occur in the previous paper, Fig. 10, when the records were led from the actual site of excitation. Assumption 2 is valid, therefore, in these experiments because when the membrane current at the excitation site has the critical value, it has nearly the same value for a millimetre or so on either side. Hence the local potential arises in conditions suitable for its immediate propagation, and we may neglect the exact locality of the nodes of Ranvier.

From this discussion it appears reasonable to accept our prime assumptions as applicable to the conditions of the experiments of this paper. The whole analysis then follows inevitably, and the results are encouraging. It has been possible to obtain the slot excitability curve by two very different methods, and to apply the curve so obtained to predict rather accurately the threshold and latency over the whole range of stimulation with two slots. If we may extend the method to any number of slots arbitrarily disposed, we shall obtain the complete solution of the problem of space distribution of excitability. Since, however, it is rather a large step to argue from experiments with two slots to conclusions about twenty, we shall postpone the general theory until this step has been strengthened by the experiments of the two papers which follow.

SUMMARY

1. The paper attempts to determine the spatial distribution of excitability in the neighbourhood of a slot electrode—the slot excitability curve.

2. Experimental results are analysed in terms of two prime assumptions given on p. 327 and discussed on p. 340.

3. Two methods are used to determine the curve; they give good agreement over the admissible range, Figs. 4 and 5(*a*).

4. If the slot excitability curve is known, it is possible to calculate for uniform nerve the space distribution of excitability for any electrode arrangement. Fig. 5(*b*)–(*f*) shows the comparison between prediction and observation over the full range where two slots of varying strength and position are used simultaneously to stimulate.

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SPACE DISTRIBUTION OF EXCITABILITY IN THE FROG'S SCIATIC NERVE STIMULATED BY POLAR ELECTRODES

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The most convenient way to stimulate a nerve is by applying the current through a pair of wires separated from each other by a few millimetres. It has long been known that the shorter the interpolar distance the greater must be the current in order to excite. This has usually been attributed to the partial neutralization of cathodal stimulation by the spread of anodal depression. With suitable assumptions, therefore, the curve of anodal spread can be found by observing the rise in threshold as the anode approaches. A plausible example of this treatment is where the result is derived from the well-known cable theory of nerve structure. If the nerve behaves like an ohmic cable and if the potential difference across the sheath must reach a critical value in order to excite, several quantitative deductions may be made, of which two may be mentioned. First, excitation will arise exactly at the cathode whatever the interpolar distance; and secondly, the excitability will fall away from this maximal point along an exponential curve. From this it follows that the excitability (=1/threshold) for interpolar distance s should have the value

$$1-e^{s/\lambda},$$

where λ is the space constant of the cable, and the excitability with large interpolar distance is taken as unity.

The first deduction above was a classical fact of nerve physiology and the second was confirmed to fairly good approximation by Rushton (1927) in experiments designed for the purpose as well as by measuring the observations of former workers. However, if we accept the simple cable explanation of the strength-length curve, we are forced to the expectation that with very short interpolar distances (as well as with greater), excitation should arise exactly at the cathode, and the excitability in the extrapolar region should fall away exponentially (with $\lambda = 5$ mm. if the nerve is immersed in Ringer's fluid). But from the preceding two papers (Rushton, 1949; Rashbass & Rushton, 1949) it is plain that neither of these conclusions is true. For excitation arises some

3 mm. away from the manifest cathode and the slot excitability curve is very different from the simple exponential required.

There is a further deduction from the cable theory which has not received attention in the past, but turns out to be important. It is the relation between the threshold with bipolar stimulation just discussed, and the symmetrical tripolar arrangement. The circuits are shown in Fig. 1 with respectively one key or both keys closed. The expectation from the cable theory is that the threshold current through electrode O will be the same whether K_y is open and all the current passes along OX, or whether both keys are closed and half the current passes along OY. This follows at once from the Superposition Theorem. For if excitation occurs at O in each bipolar case, the effect of tripolar stimulation with half the current in each branch will be to bring the excitability at O again to threshold.

Now we have reason to expect that excitation will not arise exactly at O in all bipolar conditions, thus bipolar and tripolar thresholds should not always be the same. But their divergence gives just the information required for an exact understanding of bipolar excitability, so in the experimental work, we have measured tripolar as well as bipolar thresholds, using the circuit of Fig. 1.

THE STRENGTH-LENGTH CURVE

Method. We wish to lead current into the nerve at one electrode and out at another in conditions so uniform that between the electrodes the potential gradient of the nerve surface is constant and in extra-polar regions the gradient is zero. We also require the change of gradient at the electrode to be as abrupt and well localized as possible. A good way of achieving this is to set the nerve in a uniform agar-jelly rod 1 mm. in diameter made up with Ringer. This rod may then be threaded through wire ring electrodes of exactly the right size to admit it. Details are as follows:

The sciatic nerve and peroneal branch were dissected out as usual. A piece of thick glass capillary tubing was selected with bore about 1 mm. diameter and length 8 cm. and the thread attached to the spinal end of the nerve was drawn through by a fine wire. A stiff jelly of agar in Ringer was heated to liquid and then rapidly cooled under the tap. There is a short interval between the jelly reaching 35° C. and its setting, and this time is used to draw nerve and jelly into the capillary. The liquid agar is poured into a Petri dish and the nerve is at once immersed in it. Then, with the lower end of the capillary below the surface, the thread is pulled gently so that the spinal end of the nerve with knot is drawn into the tube with some adhering jelly. This at once plugs the tube and forms a piston so that upon gently pulling the thread farther, nerve and liquid jelly enter below with no air gaps.

The whole nerve down to the knee is drawn in and the tube is placed on one side for a few minutes for the jelly to set, with precaution against desiccation of the exposed peroneal branch. The contents of the tube are then expelled by blowing (by mouth) down the upper end of the tube. The nerve set in its jelly rod is thus gently driven out into a dish of Ringer's fluid.

The electrode system is indicated in Fig. 1. Each electrode was a ring of chlorided silver wire (s.w.g. No. 34) which just allowed the jelly rod to pass. The nerve and middle electrode O were fixed, but X and Y could independently be moved by rack and pinion attachments. O was always arranged to be midway between X and Y. The whole system was in a moist chamber, and the thin layer of moisture on the surface of the jelly caused the rings to make good contact with the jelly and to slide over it easily, but there was no appreciable accumulation of fluid by surface tension between the rings even when they were as close as 1 mm.

In order to test the potential applied to the nerve surface by this arrangement when K_x only is closed, two methods were used. In the first, fine probes attached to cathode followers explored the surface of the agar rod containing the nerve. The result is shown in Fig. 2 (dots). In the second method a fine wire insulated except for the tip was set in the middle of the agar rod instead of the nerve. The potential of the tip as the rod was slid past the cathode is shown in Fig. 2 (crosses). The two curves deviate from the ideal of two straight lines in the way anticipated, but the deviation is about as small as can be expected when a whole nerve trunk is used.

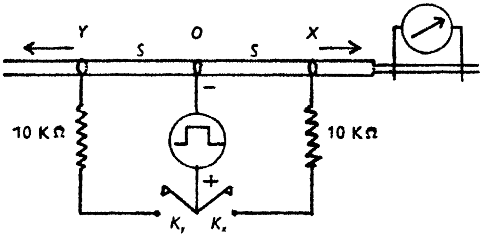

Fig. 1.

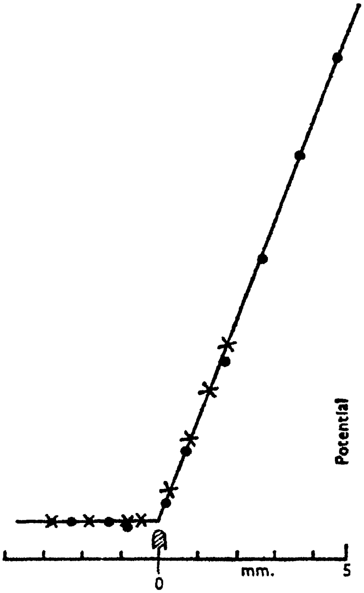

Fig. 2.

Fig. 1. Circuit for stimulating a nerve either through bipolar or symmetrical tripolar electrodes.

Fig. 2. Dots show the potential of the surface of a 1 mm. diameter jelly rod set around the nerve in the circuit of Fig. 1, where K_y was open and electrode X far to the right. Crosses show the potential in the middle of the jelly rod where an electric probe replaced the nerve. The size of the electrode wire is shown above the mm. scale at 0.

A matter which has got to be taken into consideration in strength-length measurements, is the change in resistance of the nerve resulting from alteration in interpolar distance. We have used a current generator whose output is practically independent of the output impedance. This has recently been described (Rushton, 1949*a*) and it need only be stated here that the output current is accurately proportional to the voltage applied to the grid of the input valve.

In the present experiments this voltage was a square wave of fixed duration of about 0·5 msec. and the intensity was measured with a calibrated potentiometer.

In order to satisfy ourselves that for a given setting of input voltage the current through the rod OX was independent of the distance OX, we measured this current as follows.

Two fine wire probes 1 mm. apart made contact with the jelly rod OX near O. They led via cathode followers to a system for measuring by null point the potential difference of the two probes. It was found that this potential difference was accurately the same whether X was just to the right of the probes or far to the right. Thus the current is simply dependent upon the voltage input to the generator and not at all upon the length of the stretch OX.

The current generator was useful in comparing bipolar and tripolar thresholds. Since the setting measured was proportional to the output current through O, the results give this current directly, and are unaffected by the change in circuit resistance in the two conditions of stimulation. When s is great, for instance, the two thresholds were identical.

Experiment. We require to excite with cathode at O (the mid-point between X and Y) in the three conditions obtained by closing K_x or K_y or both. For each case we measure the threshold current through O and the latency of the response. For short interpolar distances a small error in the position of the electrode makes a great change in the threshold current required. It was therefore found best to set X accurately (correct to 0·1 mm.) and find the threshold, and then to set Y to such a position that it gave the same threshold. This method generally resulted in a symmetrical setting as observed through a lens. Latencies were measured for each threshold observation, by the method described in the previous paper.

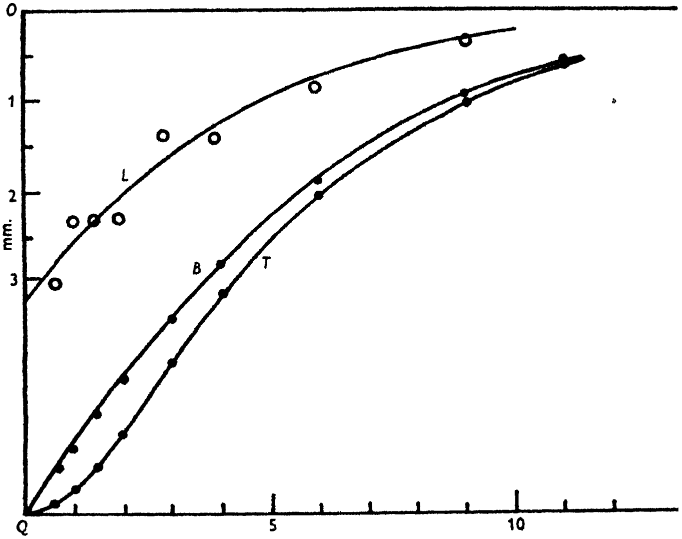

Fig. 3. Dots B and T plot excitability as a function of interpolar length for bipolar and tripolar stimulation. L plots difference in the latency of response in the two bipolar cases OX and OY. This is measured downwards from the upper horizontal giving distance of excitation site from cathode. Curves B and L are calculated from T without any arbitrary constant.

RESULTS

These are given in the three curves of Fig. 3. Curve T gives the result of tripolar stimulation, where excitability ($=1$/threshold current through electrode O) is plotted against the interpolar length s. Curve B shows the excitability with bipolar stimulation. It is the same whether stretch OX or OY is measured. But when the interpolar length is small, the latency of response is not the same for OX or OY for the impulse will arise from a little on either side of electrode O in the two cases.

The latency difference in the two bipolar cases is shown by the circles of curve L measured downwards from the upper horizontal (in order to keep the curves clear of each other). The scale on the left gives the distance of the excitation site from O (in millimetres) computed by multiplying half the latency difference by the conduction velocity (separately measured). All three curves meet the upper horizontal at great interpolar distances.

Now curve B is seen at once to be of the general form found by all preceding investigators, and not very far from the exponential

$$1-e^{-s/\lambda}$$

predicted upon the simple cable theory. However, the two other predictions which should go with it are not realized. The first is that the excitation site must always be at O, whereas curve L, Fig. 3, shows that it may lie as much as 3 mm. on either side of O. This of course corresponds exactly to the results of the previous two papers. The second prediction is that curves B and T should be identical, whereas they always show a characteristic divergence.

It is clearly misleading then to regard curve B as the expression of current distribution in a simple cable, nor shall we in this paper suggest any other structural explanation. But we shall give an analytical explanation sufficient for the quantitative prediction of relations such as those of Fig. 3. In the previous paper we were able to determine the spatial distribution of excitability about a slot, *the slot excitability curve*, and in terms of it to explain quantitatively various excitability relations by the application of the Superposition Theorem. In similar fashion here we shall determine the distribution of excitability about a single electrode, *the single-pole excitability curve*, and in terms of this explain the curves of Fig. 3 and other relations. The prime assumptions are the same as those of the foregoing paper.

THE SINGLE-POLE EXCITABILITY CURVE

One is so used to thinking that threshold excitation must arise exactly at the cathode that most of the methods which jump to mind for determining the single-pole curve are invalidated by the falseness of this assumption. Actually the method we adopt is simple but the logic which justifies it is not so simple. Let us take it in stages.

A. *The single-pole excitability curve is symmetrical.* Suppose first that the space distribution of the applied potential is that shown in Fig. 4(a). The cathode is at O and there are two anodes symmetrically placed far away to the right and left. Let the curve PQR show the distribution of excitability about O in this case. It will obviously be symmetrical about OQ. Now consider a different case, namely (b) where O is the mid-point of a very long uniform potential gradient. Since the anode and cathode are both very far away there will be no excitability at all in the region of O due to this stimulus. Thus the superposition of conditions (a) and (b) which give (c) corresponds to an excitability curve PQR plus zero. It follows that for great interpolar lengths bipolar stimulation gives rise to the same distribution of excitability about the cathode O as does tripolar stimulation. Curve *PQR* in fact is the single-pole excitability curve which we seek, and we have seen that it is symmetrical about *OQ*.

B. *The differential of the single-pole excitability curve is minus the slot excitability curve.* Let us denote the single-pole excitability curve *PQR*, Fig. 4, by

$$y = \psi(x),$$

where y is the excitability of a point distant x from O. The slot excitability curve is defined as the excitability produced by two electrodes very close together. From the Superposition Theorem this will be

$$y_1 = \psi(x) - \psi(x + \Delta x),$$

because we have shown that $f(x)$ is an even (symmetrical) function, therefore

$$y_1 = -\Delta x \frac{d}{dx} \psi(x).$$

This at once suggests that we may obtain the single-pole excitability curve by finding the slot excitability curve as in the previous paper, and integrating the result. We have preferred, however, to use another and much simpler method. In the paper which follows, however, Rashbass (1949) has obtained the two curves separately and found one to be exactly the integral of the other.

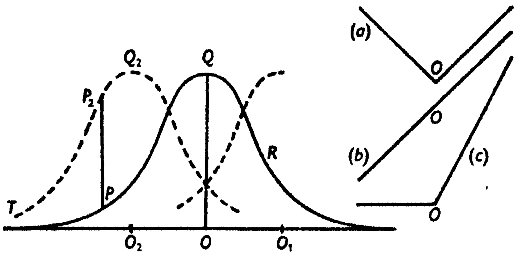

Fig. 4.

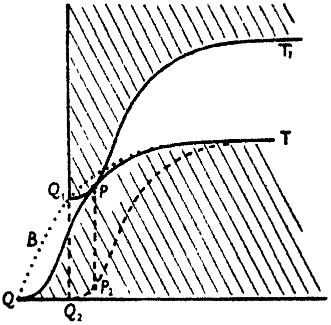

Fig. 5.

C. *In symmetrical tripolar stimulation* (*Fig.* 1) *the excitation site is always at O.* Consider Fig. 4 and suppose the anodes are at O_1 and O_2, a distance x from O. The excitability produced will thus be the sum of twice the curve *PQR* less the two dotted curves which correspond to O_1 and O_2. It is obvious that the resulting curve runs horizontal at O, hence it will be either a maximum there, or a local minimum. It will be a maximum if

$$\frac{d^2}{dx^2}\psi(o) - \frac{d^2}{dx^2}\psi(x) < 0,$$

or

$$\frac{d}{dx}\left\{-\frac{d}{dx}\psi(o)\right\} > \frac{d}{dx}\left\{-\frac{d}{dx}\psi(x)\right\}.$$

But from section *B* above, this condition is that the slope of the slot excitability curve at *O* should be greater than its slope at abscissa x. Now we have seen that the shape of the slot excitability curve is such that its slope at *O* is greater than at any other point whatever. So the condition that tripolar excitation should be a maximum at *O* is *always* satisfied. Hence in symmetrical tripolar stimulation (Fig. 1), excitation always arises exactly at *O*.

D. *Relation between tripolar and single-pole excitability curves.* From Fig. 4 it is clear that the tripolar excitability at *O* is twice $\psi(o)-\psi(x)$. But $\psi(o)-\psi(x)$ is simply the curve described by *PQR* when Fig. 4 is inverted and the origin placed at *Q*. Consequently, the single-pole curve which we require is seen already plotted (upside down) in curve *T* of Fig. 3. If then we invert Fig. 3, curve *T* together with its reflexion in *OQ* will give in full the single-pole excitability curve of the nerve, plotted with origin at *O*.

It is easy to see intuitively why *T* inverted should be the single-pole excitability curve if excitation always arises from electrode *O* (Fig. 1). For the inverted single-pole curve represents the curve of depression due to an anode at *Q* (Fig. 3). As the cathode moves farther away to the right, the excitability increases by exactly the diminution in the anodal depression.

Analysis of Fig. 3. Now that we have obtained the single-pole excitability curve *T* (inverted), the next problem is to deduce curves *B* and *L* (Fig. 3) from it. The easiest way to do this is shown in Fig. 5. The curve *QPT* is drawn on a card and cut out. The upper portion is now slid on the lower so that the vertical through Q_1 remains vertical. Then Q_1 the origin of the upper portion traces out the curve *B* and the horizontal distance from Q_1 to the contact point *P* gives the ordinate of curve *L*. In fact, in Fig. 3, the curves of *B* and *L* are not arbitrarily drawn to go through the points, but are derived from curve *T* by the foregoing construction, with nothing arbitrary at all.

The validity of the foregoing construction depends simply upon the fact that the tripolar curve *QPT* is the polar excitability curve inverted, and also upon the fundamental principle that bipolar excitability is the difference between the single-pole curves at anode and cathode. For suppose in Fig. 4, *O* is anode and O_2 cathode, and O_1 is neglected. In this bipolar case the excitability at any point *P* will be given by the ordinate difference P_2P. Now if we turn this curve upside down, curves *QPT* and Q_2P_2T are precisely as shown in Fig. 5 by the same letters, so clearly Fig. 5 may be given the same significance. Namely an anode at *Q* and a cathode at Q_2 will result in an excitability shown by the ordinate difference of *QPT* and Q_2P_2T. This is maximal at *P*, the contact point, and the difference PP_2 is equal to Q_1Q_2. It follows that the co-ordinates of Q_1 give the interpolar length and corresponding excitability, and the excitation site, *P*, lies the horizontal distance Q_1P from the cathode. The success of this analysis in accounting for the results of Fig. 2 is obvious.

Note upon the latency measurements

The curve B, Fig. 3, is reliable, and we have nearly always obtained a fit as good as this. But the latency measurements plotted happen to be exceptionally favourable to the theory, and with this particular nerve the latency for tripolar stimulation indicated a site variation of 1·5 mm. when, as we have argued, it should remain fixed at O. This latency is not very consistent during a set of measurements and the direction of the deviation from the theoretical expectation varies in a random manner from one preparation to another. For this reason it was impossible to base the important conclusion that curve T always excited at O upon actual latency measurements for in fact those generally showed that it did not. We therefore had recourse to the theoretical argument which proved that in uniform nerve it should do so. It is now proper to consider whether the non-uniformity which produced the latency variation is likely also to invalidate the theoretical treatment employed.

Consider first the bipolar curve constructed according to the method of Fig. 5. When the interpolar length is not too small the contact point P is sharply defined, and we find experimentally too that in this region the latency carries no ambiguity. But when Q_1 is near to Q the two curves lie in close proximity over a considerable extent, and a very slight irregularity of local excitability along the nerve can shift the actual point of contact 1 or 2 mm. without appreciably affecting the threshold current. For this reason the excitability measurements remain almost exactly as calculated upon the assumption of uniform nerve despite an appreciable latency variation. The same kind of consideration applies in the case of tripolar excitation, except that here it is with small interpolar lengths that the excitation site is most sharply defined.

We therefore expect good agreement with the theory in our excitability measurements, and are not surprised to find in latency, 1 mm. or so divergence from the predicted site.

Tripolar stimulation with X and Y at different potentials

The tripolar stimulation so far considered has been symmetrical about the cathode. The polar excitability curve so obtained may be used to predict the more complicated asymmetric cases. One class of these will now be considered because the correspondence between prediction and observation strengthens the concepts of these papers, and because the matter can be presented very briefly.

In the examples to be considered, the three electrodes remain fixed, one midway between the other two, but the currents in the two interpolar stretches of nerve are not equal.

For each arbitrary current through electrode X, the current through Y is found which just brings the excitation to threshold, and the current strengths X and Y are plotted one against the other in the usual manner. Both method and result are somewhat similar to Part III of the preceding paper.

Experiment. This was carried out exactly as in the former paper, except that the electrodes were not slots but the Ag-AgCl rings applied to the jelly round the nerve. In series with each electrode X and Y was placed a 1000 Ω resistance, the potential drop across which was used in calibrating the plotting cylinder. Latency measurements were taken as before, and converted into distances by measuring the nerve conduction velocity.

RESULTS

These are shown in Fig. 6. They are taken from the same nerve as gave the results of Fig. 3. In each case only the curves above the 45° line are shown, the symmetrical lower half is suppressed and replaced by the theoretical curve. This was predicted from the tripolar curve T, Fig. 3, by a method altogether analogous to that in the previous paper. As Fig. 6 shows, the result is a family of lines each corresponding to one determined excitation site.

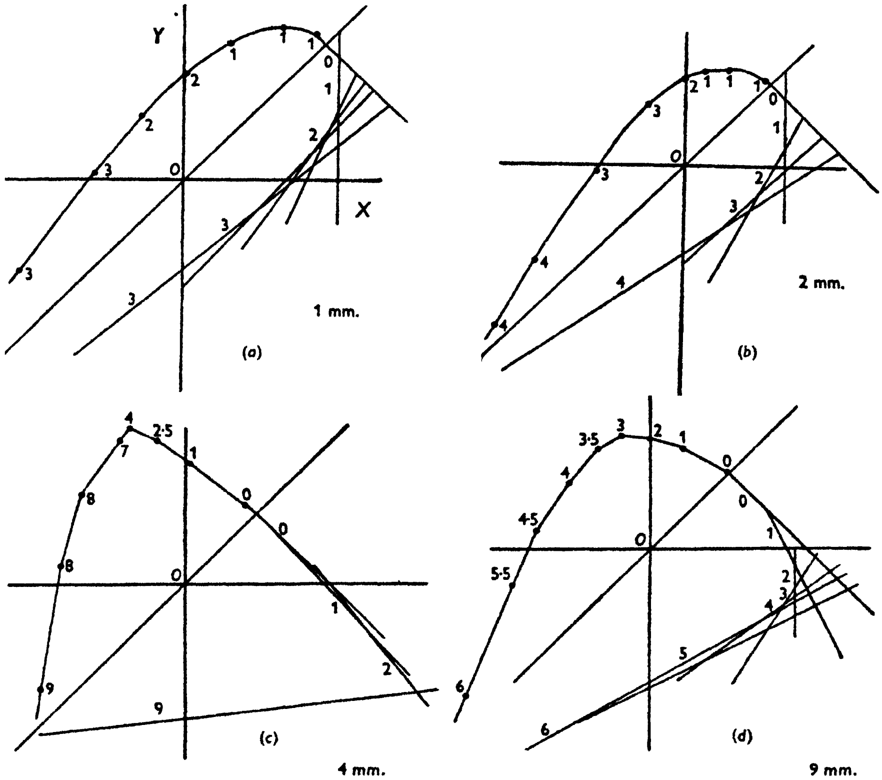

Fig. 6 (*a–d*). Results of stimulating with tripolar electrodes (see Fig. 1) with distance $OX = OY = 1$, 2, 4, 9 mm. respectively. Current through X plotted against Y for threshold. Numbers against each point show distance (mm.) of excitation site from middle electrode. This is found from response latency and conduction velocity. Only the experimental points above the 45° line are shown. Below are the theoretical results calculated from Fig. 3, curve T (same nerve). The envelope and numbers should be a reflexion in the 45° line of the observed results.

It is clear that the observed curve corresponds well enough with the predicted envelope of lines and also with the corresponding excitation sites. As in the former case, observation diverges from prediction when interpolar distances increase, for it is impossible to secure a uniform threshold over so large a stretch of nerve. But on the whole the correspondence is satisfactory enough. With distances greater than 12 mm., observation and theory show exact triangles.

Former work. Rushton (1928*b*) used only large interpolar lengths (15 mm.) and hence obtained simply triangles. These he interpreted as we have done here. Tasaki (1939) also found triangular results using single-nerve fibres from the frog's sciatic and interpolar stretches of one internodal length. This is quite contrary to our observations upon the whole nerve trunk, where at 1 or 2 mm. the results are far from triangular (see Fig. 6*a*, *b*).

This difference between the single fibre and the whole nerve trunk will be considered in a later paper when we attempt to explain the structural nature of the space distribution of excitability, for we believe that the novel features in our views on excitability distribution do not apply to isolated fibres.

DISCUSSION

In this group of papers we have made contributions of two kinds. In the first place our observations upon the spatial relations of excitability have been more accurate and of wider scope than those previously reported. Secondly, the theory advanced adequately describes all the observations and presents certain novel features. Previous work in this field is scanty, and since Rushton (1928*b*) we have found only Ozorio de Almeida (1931) and Rushton (1934) who confirm the general shape of the bipolar strength-length curve, and Hodgkin (1937) who obtained a relevant and consistent result by stimulating with two pairs of electrodes and observing how threshold depended upon cathode separation. Though our observations confirm and extend these results, our interpretation is different.

Former workers have always argued from the assumption that threshold excitation arises exactly at the cathode. We started by showing this assumption in general to be false, and were able to interpret observations without it. The resulting theory has one great drawback for it is incompatible with the simple cable theory which Rushton (1934) and Hodgkin (1937) found in good agreement with their interpretation, both physiologically and physically. It is beyond the scope of this paper to discuss what assumption as to the structure of the whole nerve trunk is appropriate to the new results, and without a theory of structure we cannot use the excellent observations available upon the physics of nerve (spread of electrotonic potential, etc.). But though our theory cannot *explain* the spread of excitability in nerve, it does describe it quantitatively for all the cases we have observed, and we believe that it is applicable to any distribution whatever. We shall, therefore, conclude by indicating how the excitability at every point on a uniform nerve may be predicted in the most general case. The solution is similar to that given by Rushton (1928*a*), but we now avoid the errors which result from the simple-cable assumptions.

General treatment. Suppose the stimulus applied to a uniform nerve has the space distribution of potential shown by the irregular curve of Fig. 7, then, given the single-pole excitability curve it is required to find the excitability of every point on the nerve due to this stimulus. The curve of Fig. 7 may always be broken up into a number of approximately straight lines of gradients $g_0, g_1, g_2, \ldots$

Then a current proportional to g_1 flows from x_2 to x_1, g_2 from x_3 to x_2 and so on. The condition is therefore identical with that arising from a set of electrodes applied at points x_1, x_2, x_3 ..., through which currents pass of magnitudes respectively (g_1-g_0), (g_2-g_1), (g_3-g_2), ... The single-pole curve due to each electrode may then be drawn suitably displaced and scaled, and the excitability of the nerve at every point will be simply the sum of the ordinates of all these curves.

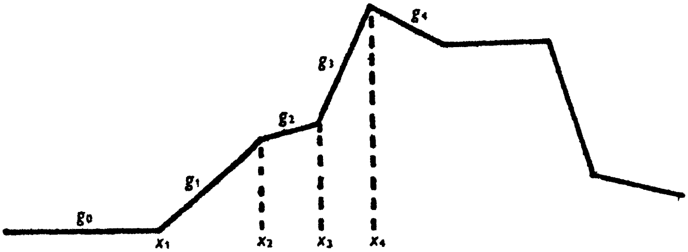

Fig. 7.

SUMMARY

1. Threshold and excitation site are determined when a nerve is stimulated through two or three electrodes.

2. A method is described of setting a nerve in a 1 mm. diameter agar-Ringer rod which makes the applied potential very uniform (Fig. 2).

3. The bipolar excitability is greater than symmetrical tripolar excitability, at the same interpolar length, and when this is small the bipolar excitation arises 3 mm. extrapolar to the cathode (Fig. 3). Both these results are contrary to the simple cable theory, but fit the observations of the preceding paper.

4. By applying the Superposition Theorem and little else, the curves *B* and *L* of Fig. 3 are calculated from *T* without arbitrary constant. The deviations from the cable expectations fall therefore precisely as predicted.

5. Fig. 6 shows the comparison between observation and prediction in some examples of tripolar stimulation.

6. The theory is generalized to predict the distribution of excitability in any case where the distribution of stimulus is known.

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THE RELATIONSHIP BETWEEN THE SLOT EXCITABILITY AND THE EXCITABILITY DUE TO A SINGLE POLE

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The foregoing papers have shown how to determine the spatial distribution of excitability along a frog's nerve due either to a single-pole electrode, or to a slot (which is the equivalent of two single electrodes placed close together). Now these two distributions should be related in a simple manner, for in the previous paper (Rashbass & Rushton, 1949*b*, p. 343) it was deduced from the Superposition Theorem, that the single-pole excitability should be minus the integral of the slot excitability. The purpose of the present paper is to find out how far this theoretical expectation is experimentally true.

METHOD

The single-pole excitability was measured as in the previous paper, but the method of measuring the slot excitability was modified so that the determination could be done without removing the nerve from the jelly cylinder or from the trough in which the single-pole measurement had been made.

The circuit connexions for determining the slot excitability by this method were the same as for plotting the asymmetric tripolar curves (previous paper, p. 345) except that a variable resistance (0–50,000 Ω) was introduced in series with electrode *Y* (Fig. 2*a*). The convention for signs in plotting was the same as usual, *x* being positive when electrode *X* is positive to electrode *O*. Similarly, with *y* and electrode *Y*. Electrodes *X* and *O* remained fixed throughout the experiment, at a separation of not more than 1 mm. They constitute the slot.

Fig. 1 shows the potential measured by a probe inside the jelly cylinder when the electrodes were 1 mm. apart. It is seen that the change of potential is limited to a very short region and in fact the curve is almost identical with that obtained with a true slot (Rushton, 1949, Fig. 5).

Electrode *Y* was applied at various known distances from *O*, and in each position the following portions of the complete tripolar curve (Fig. 2*b*) were plotted. *A*, the region where the curve crosses the negative *y* axis; *B*, the point where the curve crosses the positive *x* axis; *C*, the region where the curve lies farthest to the right. Owing to the asymmetry of the electrode system it was not possible to compare the scales of the two axes by means of any physical measurement. Instead, the thresholds for stimulating through *XO* and through *YO* were defined as being equal. By adjusting the variable resistance in series with *Y* it was possible to represent these thresholds by approximately equal intercepts on the axes. Although theoretically not necessary, this adjustment rendered the records easier to plot and more accurate to analyse.

Theoretical considerations

The extraction of the slot excitability curve from these measurements was based on the following theoretical considerations. So long as the distance YO is not too great, the slope of the curve of Fig. 2*b* varies continuously, except in the third quadrant. Therefore there must be a point C on the polar curve where the tangent is parallel to the y axis (and the value of x there is a maximum). Since at that point the strength of x is independent of y, the slot electrodes must be stimulating at a point on the nerve where the current through Y is producing zero excitability. Assuming the nerve to be uniform this point will be midway between Y and O. It follows that the abscissa C gives the threshold when the slot excites a distance $\frac{1}{2}XY$ from its centre. Also OB is the threshold when the slot excites at its optimum point. Thus the ratio of the abscissae x_B/x_C will give the ordinate of the slot excitability curve corresponding to abscissa distance $\frac{1}{2}XY$, and scaled so that the maximum ordinate is unity.

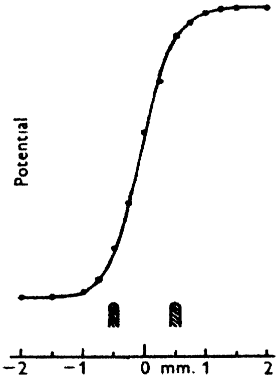

Fig. 1.

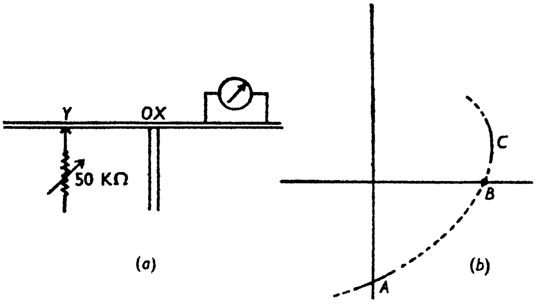

Fig. 2.

Fig. 1. The potential inside an agar jelly cylinder surrounded by two ring electrodes 1 mm. apart.

Fig. 2. (*a*) The electrode system for measuring the slot excitability curve. (*b*) The asymmetric tripolar curve showing the parts actually plotted as continuous lines.

As the distance OY is increased the shape of the polar curve changes, and eventually the slope becomes discontinuous where the point of excitation jumps from near the slot to near Y. Under these circumstances the nerve is never stimulated midway between O and Y and the above method becomes inapplicable. However, it is certainly valid for determining the slot excitability curve from the origin up to the maximum ordinate. To plot the curve for distances greater than this, use is made of portion A of the tripolar curve (Fig. 2*b*).

On the negative y axis the nerve is stimulated at or near Y (Fig. 2*a*) and the cotangent of the angle which curve A makes with that axis is proportional to the ordinate of the slot excitability curve at the point where excitation is occurring, as has been shown in a previous paper (Rashbass & Rushton, 1949*a*). Now the site of excitation is exactly at Y only when the distance OY is great. For shorter distances it is displaced into the extrapolar region and its exact location can be found by sliding the single-pole excitability curve on itself as has been shown previously (Rashbass & Rushton, 1949*b*, Fig. 3). When Y is very close to O the excitation site practically coincides with the optimum of the slot excitability curve. As Y moves away, the site also moves out to a known extent, and finally coincides with Y. Clearly then, by measuring the cotangents for each position of Y, the slot excitability curve may be found for all distances greater than the optimum.

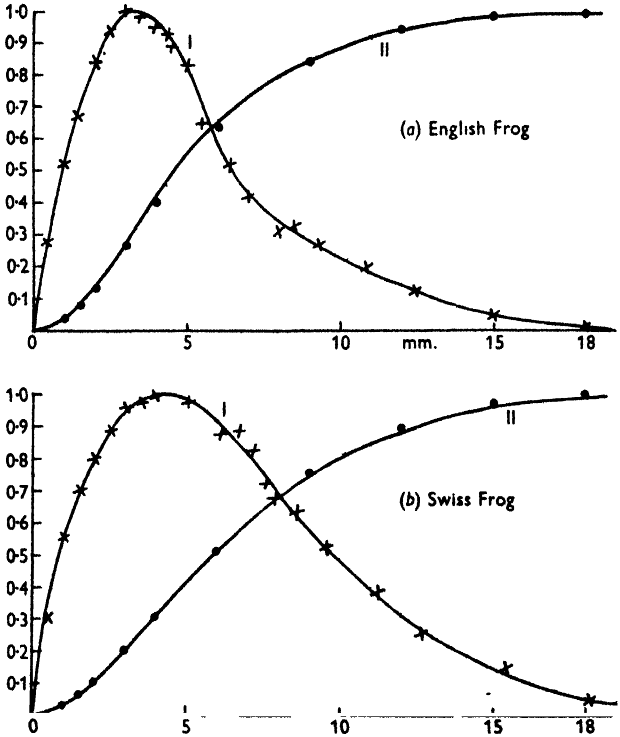

Fig. 3. I is the slot excitability curve drawn freehand through the experimental points (crosses). II is the integral of I, and the dots are the symmetrical tripolar excitabilities. All the curves are scaled to the same maximum ordinate. (*a*) Small English frog; (*b*) large Swiss frog.

By combining these two analyses, therefore, the slot excitability curve can be obtained in its entirety, and the method is not only rather accurate, but also admits of exact comparison with the single-pole excitability curve.

RESULTS

Figs. 3(*a*) and (*b*) show results obtained from the sciatic nerves of English and Swiss frogs respectively (*Rana temporaria*). The slot excitability results are shown by crosses, and curve I has been drawn freehand through them. Curve II is its calculated integral scaled to a maximum value unity. The experimental points on the symmetrical tripolar curve which is the single-pole excitability curve inverted (dots) are also scaled to a maximum of unity.

The good agreement between curve II and the dots substantiates the expectation that the slot excitability is minus the differential of the single-pole excitability.

SUMMARY

1. A method is described for determining the slot excitability curve in the same apparatus as the single-pole excitability curve.

2. The spatial distribution of excitability about a slot is shown to be minus the differential of the spatial distribution of excitability about a single pole.

I should like to thank the Medical Research Council for defraying the cost of this work with a Research Training Grant.

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INSTABILITY OF RESPONSE TO STIMULATION OF THE SENSORIMOTOR CORTEX OF MAN

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(*Received* 1 *November* 1948)

The principal contributions of Sherrington and his pupils to the study of the functions of the cerebral cortex were three: the mapping of the excitable motor area, the study of inhibition and the investigation of instability of response to stimulation. The first of these was a problem of localization, the other two were directed toward an understanding of the mechanisms involved in the utilization of the motor cortex in the performance of skilled movement.

In man, as in experimental animals, the response to stimulation of a single small volume of cerebral cortex is remarkably stable. In experimental animals variations in the conditions of stimulation do, however, give rise to differences in response. This instability of response has been rather fully investigated in animals. Observations of this kind on the cortex of man are few. The purpose of this paper is to record observations of instability of response to cortical stimulation in man.

Facilitation and extinction. Bubnoff & Heidenhain (1881), from their experience with cortical stimulation in dogs, pointed out that subliminal stimuli can become effective if repeated rapidly. This phenomenon they called summation of stimuli. These same observations were later made and extended in primates by Sherrington and his pupils and the phenomenon was named *facilitation*. It can be defined as enhancement of response to cortical stimulation, due to previous stimulation. This antecedent stimulation may be of the same or a different cortical point. In the latter case it is called *secondary* facilitation.

By local freezing and cortical incision experiments, Graham Brown (1915*a*, *b*, *c*, *d*; 1916*a*, *b*) was able to demonstrate that the essential neuronal mechanism responsible for secondary facilitation was situated in the cerebral cortex. He showed that primary facilitation was altered by the test stimulus and that it increased with repetitive stimuli only to reach a maximum and then decrease. Under the conditions of his experiments its duration, following a single stimulus, was about 10 sec., although there was variability. Since primary facilitation could be demonstrated upon stimulation of the white matter, it must, he concluded, be localized in part, at least, in subcortical mechanisms. Dusser de Barenne & McCulloch (1939) have shown locally increased pH and negative slow voltage drift to accompany this process.

McCulloch (1944) has pointed out that there are two types of secondary facilitation. One is characterized by enhancement of the usual response to the priming stimulus when test stimulation is carried out (*ab A*). In the second type the response characteristic of the test stimulation point is augmented upon test stimulation (*ab B*). The second, only, he localized to the cerebral cortex.

Secondary facilitation has been described in man by Penfield & Boldrey (1937, 1939).

Extinction, the opposite of primary facilitation, described by Dusser de Barenne & McCulloch (1934), is a decreased response to cortical stimulation, due to previous stimulation of the same point. It follows facilitation and is accompanied by opposite changes in pH and slow voltage. The specificity of this process has been questioned by Moruzzi (1939) and others who consider it to be no more than fatigue due to after discharge.

Reversal and deviation of response. These phenomena constitute special examples of instability, as observed and singled out for study by Graham Brown & Sherrington (1912) and Leyton & Sherrington (1917). Thus, although stimulation of a certain cortical point may ordinarily give rise to extension of a member, preceding stimulation of the same or another point may so alter the mechanisms involved that flexion rather than extension occurs when the test stimulus is applied. This *reversal of response* is attributed by Dusser de Barenne & McCulloch (1939) to facilitation at a spinal level and to reciprocal innervation. *Deviation of response* means the eliciting of a response of a different part than the one usually characteristic of a cortical point, due to antecedent stimulation. Deviation is especially interesting because it can be carried across functional boundaries (face to arm).

METHOD

The following observations upon the human cerebral cortex were made during the course of operations carried out as treatment for focal cerebral seizures and for cerebral tumour. Local analgesia was utilized except in case 2. This operation was performed under pentothal anaesthesia.

OBSERVATIONS

Case 1. *Primary facilitation of sensory and motor responses.* C.K. This 31-year-old man had focal cerebral seizures as the result of a gunshot wound of the right frontal lobe, sustained in battle in August 1944. Right frontal craniotomy was carried out 2 December 1947. Stimulations were made at 1 V., 60 cyc., utilizing the Rahm stimulator. Stimulation of points on the cortex as marked on Fig. 1 gave the following responses:

Point 2. Stimulation at point 2 caused slight tingling in the left thumb although previous stimulation, less than 10 sec. before, had given no response.

Point 3. Stimulation here gave no response but when this was repeated 3 sec. later there was tingling in the lower lip on the left.

Point 4. Initially there was no response to stimulation here. Restimulation 6 sec. later caused a tingling on the left side of the tongue.

Point 5. This point was stimulated without response and restimulated 9 sec. later, again without result. However, 5 sec. later stimulation of this point caused tingling in the left side of the tongue, more posterior on the tongue than that caused by stimulation at point 4.

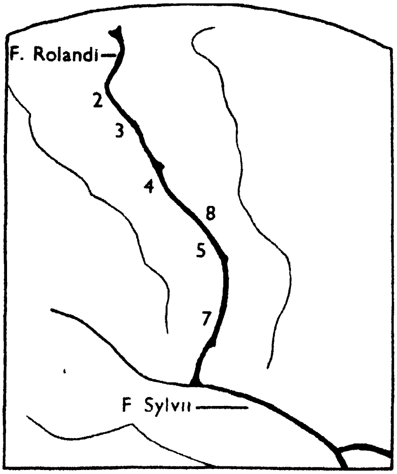

Fig. 1. Case 1. C.K. Points of stimulation on the margins of the fissure of Rolando of the right hemisphere as explained in text.

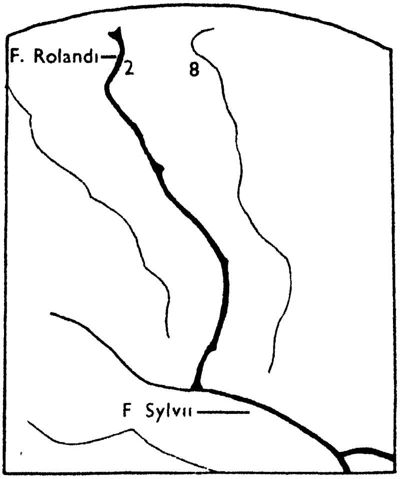

Fig. 2. Case 2. H.W. Stimulation points on the right precentral gyrus as explained in text.

Point 7. When first stimulated this point was unresponsive. This was repeated 6–8 sec. later (with two negative stimulations at another point in the interval) and tingling in the tongue was reported.

Point 8. There was no response when this point was first stimulated. When restimulated 4–5 sec. later, however, quivering was noted in the lower lip and the patient observed that it was hard for him to speak.

Case 2. Secondary facilitation of motor response and epileptic response. H.W. In the case of this 58-year-old woman, right osteoplastic craniotomy was performed 6 February 1948, and a large meningeal fibroblastoma of the falx was removed. Stimulations were carried out after removal of the tumour while the patient was under pentothal anaesthesia. The Rahm stimulator was utilized at 1 V., 60 cyc. Stimulation at points on the cortex as marked in Fig. 2 gave the following results:

Point 8 was not excitable when first stimulated. Wrist flexion and pronation occurred when point 2 was stimulated. The electrode was carried anteriorly in short steps so as to give repeated stimulation of the cortex, beginning at point 2 and ending at point 8. Each stimulus was followed by wrist flexion and pronation until point 8 was reached, when there was a local seizure beginning with the same movement.

When, after an interval of time, point 8 was restimulated no response occurred. Repeated stimulation carried out along a path from point 8 to point 2 gave no observable response until point 2 was reached, when another short seizure involving wrist flexion and pronation occurred. After 2 min. the simple movement, without after discharge, was elicited when point 2 was restimulated.

Case 3. *Extinction of motor response.* F.R. A left craniotomy was carried out 30 October 1935, for focal seizures. Stimulations were made with the Thyratron stimulator set at 30.

When the responsive sensorimotor cortex was mapped out stimulation of a certain point on the precentral gyrus caused flexion of the hand. The effect of preceding stimulation of this point upon later stimulation of the same point was observed. The response to the test stimulus was abolished three times out of four trials when the interval between stimulations was 4 sec. and once when it was 6 sec.

Case 4. *Secondary facilitation of sensory responses. Sensory inhibition. Deviation of sensory responses.* M.C. Operation on this young woman was carried out 13 February 1948, for an oligodendroglioma in the right frontal lobe. Stimulations were made with the Rahm stimulator at 1 V., 60 cyc. The following results were obtained by stimulation at points shown in Fig. 3.

Stimulation of point 18 caused tingling of the left little finger and extension of the finger. At point 19, tingling and flexion of the fingers was produced. When the electrode was made to touch the cortex at short intervals along a path from points 18 to 24 and then to point 23, there was sensation in the fingers at each stimulation until point 23 was reached when it was felt in the palm. Points 23 and 24 when stimulated previously had given rise to hand sensation. Again, repeated stimulation across the gyrus from points 19 to 23 caused finger sensation all the way. On inquiry she admitted experiencing slight palm sensation as well when 23 was reached.

This then is an example of deviation of sensory response.

Case 5. *Primary and secondary facilitation of sensory response. Sensory inhibition. Deviation of sensory response.* H.E. This 51-year-old man had a left osteoplastic craniotomy 4 March 1948, for focal cerebral seizures of 17 years' duration. Stimuli were made at 1 and 1½ V., 60 cyc., utilizing the Rahm stimulator. Stimulation at points illustrated in Fig. 4 gave the following responses:

Point 4. Stimulation at point 4 gave rise to a prickling sensation at the tip of the tongue. Almost 2 hr. later this point was stimulated without response. This was repeated at 1 sec. intervals until the third stimulus caused tongue sensation. The stimulating electrode was then moved posteriorly in short steps toward point 35 and tongue sensation was reported at each stimulus until point 35 was reached, when it was no longer produced.

Point 15. Stimulation at point 15 caused sensation at the tip of the right middle finger.

Point 17. When point 17 was stimulated there was a sensation on the radial aspect of the index finger. By moving the electrode posteriorly in steps this same sensation was produced from points between 17 and 38. At point 39 the sensation was reported as index finger and thumb. Then he complained of tingling in all fingers. This persisted for 1 min. after withdrawal of the electrode.

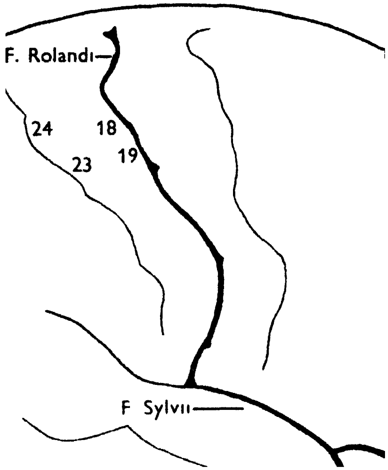

Fig. 3. Case 4. M.C. Stimulation points on the right postcentral gyrus as described in text.

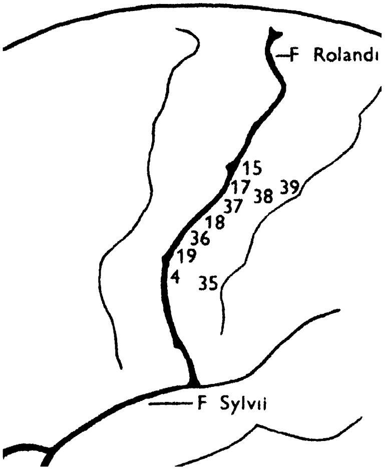

Fig. 4. Case 5. H.E. Stimulation points on the left postcentral gyrus as described in text.

Point 18. A sensation on the dorsum of the thumb occurred when point 18 was stimulated.

Point 19. When point 19 was stimulated the patient pointed to the lower right teeth.

Point 36. Stimulation at point 36 gave no response. On repetition there was sensation in the lower lip. Progressive stimulations were made upward through point 18 to a point just below point 37, each stimulus producing lower lip sensation. Thus the usual response to stimulation of point 18 (sensation in thumb) underwent deviation.

Point 37. Stimulation at point 37 gave no response. When this was repeated right thumb sensation was reported. The electrode was carried downward in short steps through points 18–36, each stimulus causing thumb sensation. Just below point 36 thumb sensation was produced together with lip sensation. Thus the ordinary responses to stimulation of points 18 and 36 underwent deviation and the facilitated response was made to cross a functional border (arm to face).

DISCUSSION

Observations of instability of response to stimulation, with the exception of secondary facilitation, have hitherto been confined to motor phenomena in experimental animals. The present observations indicate that instability of response is a property of the human central nervous system as well, and that it applies to sensory as well as motor responses.

Primary and secondary facilitation of motor responses in man seem to differ in no way from those described in other species. Although its duration, under the conditions in which these observations were made, is not known, it is more pronounced at short intervals of stimulation. Both types of secondary facilitation described by McCulloch (1944) (*ab A* and *ab B*). can be demonstrated upon stimulation of the motor cortex of man.

Facilitation of sensory response cannot, of course, be demonstrated in infrahuman species, although Adrian (1936) has shown facilitation of electrical response to stimulation of the sensory cortex as well as other areas in the rabbit. Sensory facilitation in man resembles motor facilitation in that both primary and secondary forms are demonstrable. It should be emphasized that secondary facilitation of sensory response, as we have seen it, has always been augmentation of the usual response to the conditioning stimulus upon test stimulation. It is this type of facilitation which McCulloch (1944), from data obtained by stimulating the motor cortex, localized in the spinal cord. This cannot be the case in sensory secondary facilitation, but the events responsible for secondary facilitation of sensory response may take place at a supracortical level of sensation.

It has been possible to demonstrate extinction in the human motor cortex. The data presented do not justify any discussion of the specificity of this process or its differentiation from fatigue due to local after discharge.

Deviation and reversal of response are special cases of facilitation within its definition. In each instance, however, there is not only an augmentative effect but also the usual response to the test stimulation point (the response deviated or reversed) is abolished. When reversal of response takes place upon proper stimulation of the motor cortex, the negative phase is accounted for on the basis of reciprocal innervation. The inhibitory or negative phase seen in deviation of sensory response cannot be accounted for on this basis. Instead it must represent a specific inhibitory effect from stimulation of one sensory point upon the response to stimulation of another sensory point. We have called this phenomenon *sensory inhibition*. The conditions under which sensory inhibition can be elicited have not yet been completely defined. Its localization, although probably subcortical, is not known and the mechanism of its production has not been elucidated.

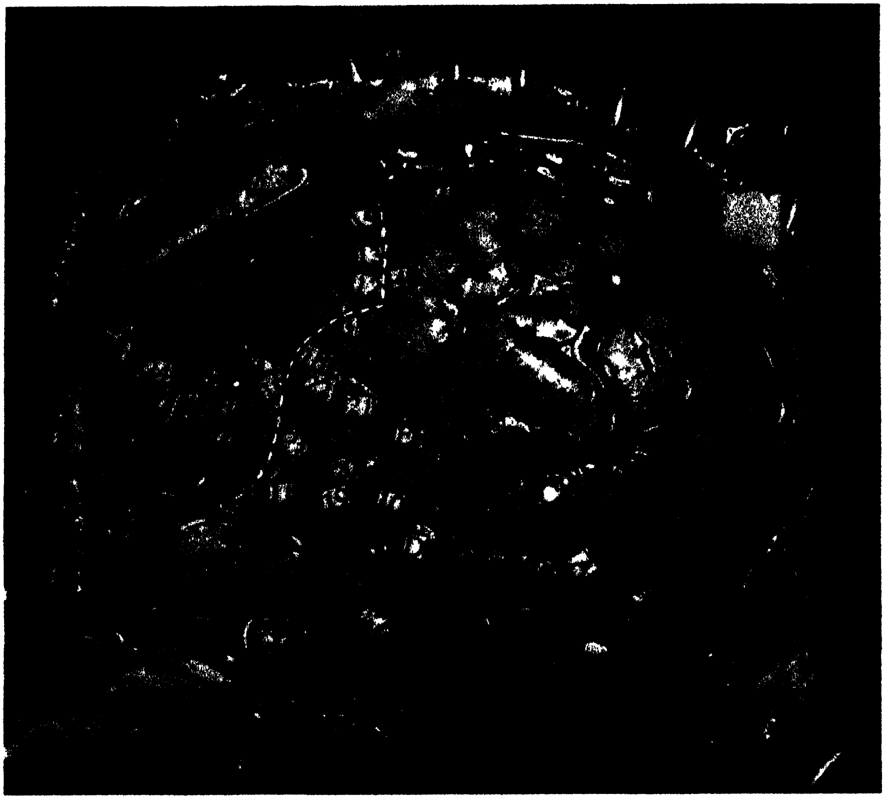

Fig. 5. J.M. Exposed left hemisphere of patient who had masticatory seizures. The central sulcus is marked with an interrupted white line. Mastication was produced by stimulation at points 19, 20, 22, 23, at a point between 11 and 19, and at a point on the small gyrus anterior to the precentral gyrus at its lowermost portion. The zone for the elicitation of this response is much larger than that usually seen.

Facilitation probably accounts for the widening of the excitable zone for the elicitation of initial phenomena of seizures upon stimulation of the cortex in patients having focal seizures. Fig. 5 demonstrates an enlarged masticatory area in a patient who had seizures beginning with mastication. A discharging epileptic focus seems to have caused an enlargement of the region from which chewing movements could be produced upon electrical stimulation. Furthermore, the conditioning effect of seizures upon the excitability of the temporal lobe (Penfield & Boldrey, 1939) may have a similar basis, although other factors must operate here as well.

Sensory inhibition and facilitation afford mechanisms for the analysis of sensory experiences, in particular for the integration of the spatial aspects of sensation. They must be of importance in the discrete localization of peripheral stimuli.

SUMMARY

1. Instability of response to stimulation of points in the sensorimotor cortex of man has been demonstrated:

(*a*) Motor responses to stimulation of the precentral gyrus have shown primary facilitation, secondary facilitation of both varieties and extinction.

(*b*) Sensory responses to stimulation of the postcentral gyrus have shown primary facilitation, secondary facilitation of one type (*ab A*), sensory inhibition and deviation.

2. The significance of these observations has been discussed.

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THE EFFECTS OF COOLING AND OF VARIOUS MEANS OF WARMING ON THE SKIN AND BODY TEMPERATURE OF MEN

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A series of experiments by Uprus, Gaylor & Carmichael (1935, 1936) and Uprus, Gaylor, Williams & Carmichael (1935) has shown that there is little uniformity in the way in which the human body warms up after cooling. These authors also suggested (1936) that warming by air which was at a higher temperature than that of the body was insufficient to raise the rectal temperature of men who had been previously cooled, and a similar view was reached in wartime experiments reported by Alexander (1945) and again by Adolph & Molnar (1946). Uprus and his colleagues have also shown that an adequate rise of the rectal temperature can be obtained after moderate cooling if part of the body is immersed in hot water, and the experiments reported by Alexander (1945) have shown that death from extreme cooling is best prevented in both animals and men by rapid immersion of the whole body in hot water at about 50° C.

People, however, frequently get cold, and they seem to warm up quite well without taking a hot bath, merely by sitting in a warm place, by exercise, or by taking hot food and drinks. It seemed worth while to study these apparent contradictions both from their practical and physiological aspects, and it was decided to investigate how men whose rectal temperature had fallen by about 1° C. would warm up under the following conditions:

(*a*) Rest at room temperature.
(*b*) A drink of hot sweet cocoa, followed by rest at room temperature.
(*c*) Alternate periods of exercise and rest at room temperature.
(*d*) Rest at about 30° C.

METHODS

Subjects. Eight intelligent and healthy sailors aged 19–25 years were selected, but one of them had to be excluded after 2 days on account of a feverish upper respiratory infection. The area of their body surfaces varied from 1·76 to 1·88 sq.m. Two of the subjects had been working in hot humid air for 3 hr. a day for 5 weeks before the present investigation began, and they must be considered to have been acclimatized to heat (Robinson, Turrell, Belding & Horvath ,1943; Bean

& Eichna, 1943). Four subjects had come straight from a cold naval base where they had been doing outdoor duties and sleeping in unheated huts, while two subjects had not been exposed to any extremes of temperature during the last months before this experiment.

Procedure. The subjects were tested in two groups of four, the first while outdoor temperatures were 4–9° C., the second at outdoor temperatures of −3 to +4° C. After 2 days the first group was reduced to three by the indisposition of one subject. Each group was tested on four successive mornings, but the experiments on the second group were carried out some weeks after those on the first. The subjects wore cotton vests and pants, woollen jerseys, worsted trousers, woollen socks, and leather shoes. They lived together. They got up and had breakfast at the same time every day, each man eating the same type of breakfast and roughly the same amount of food on each day of the experiment. About 30 min. after breakfast they walked about $\frac{3}{4}$ mile to the laboratory where the tests were being performed. They sat still at 17° C. (±2° C.) for 15 min., and then their skin and rectal temperatures were measured. Simultaneous mouth temperature measurements were made on two subjects in each group. For the following $1\frac{1}{2}$ hr. they sat in a cold room at 0° C. (±1° C.). After that one subject sat still in a room at 29° C. (±$1\frac{1}{2}$° C.) and about 50% saturation with moisture, and the other three in a room at 17° C. (±2° C.). Two of the latter sat still all the time, but one of them drank one pint of hot sweet cocoa as soon as he left the cold room; the last man stepped up and down on to a chair 30 times in 60 sec. every 15 min. The cocoa was drunk at a temperature of 45–50° C.; the sugar and cocoa powder in it were equivalent to about 200 cal. (McCance & Widdowson, 1946). Skin, rectal, and in four subjects also mouth temperatures were measured at 15 min. intervals throughout each test beginning 15 min. after entering the cold room. (Allowance was made for the 2–3 min. spent walking between the cold room and the laboratory or the hot room). Exercise, if taken, followed immediately after the measurements, so that the latter were always made in the sitting posture after a period of rest. Each subject was warmed in a different way on each day of the experiment; complete 'crossing over' was thus achieved within each group.

The first set of measurements was always taken about 1 hr. after a meal, but this need not have had any effect on the initial readings, for vasomotor responses to food appear to be slight at temperatures below 20° C. (Richards, 1946). The subjects, furthermore, walked to the experiment each morning, and they walked about 200 yd. across the grounds to and from the cold room, but the influence of all these possible sources of irregularities was excluded by their constancy from day to day.

Skin temperature measurements. The skin temperature was measured in the centre of the forehead and over the ball of the left thumb. When the subjects sat still, they always rested their left hand and forearm in a horizontal position with the thumb uppermost. The face varies its temperature over a narrower range than the limbs (Bedford, 1935), but it reacts more rapidly (Loewy & Dorno, 1925); the hand, moreover, reacts to changes of temperature more readily than the foot (Pickering & Hess, 1933) Thus, if a general pattern of vasomotor reactions is sought, this can be shown by two carefully chosen points, one on the face and one on a limb.

Difficulties in measuring skin temperatures can be caused by the fact that thermometers are either held in position by some insulating material or protrude freely (Bedford & Warner, 1934). Ordinary skin thermometers can also prevent or enhance the evaporation of moisture from the skin. Radiation thermopiles have been alleged to be free from these disadvantages (Bedford & Warner, 1934; Hardy, 1934; Stoll & Hardy, 1948), but under experimental conditions they can become cumbersome and inaccurate tools (Tobias, Loomis, & Lawrence, 1947). It appeared however, that these difficulties could be overcome if very thin wires were used to make up a thermocouple and if those wires were in close apposition to the skin over a sufficient distance to ensure that their junction completely took up the temperature of the body surface without changing it and without being itself influenced by the environment. This purpose was achieved by placing the junction and its leads loosely across the arms of a glass Y tube (Fig. 1). Estimations of the skin temperature could then be made in such a way that the junction lay over the exact spot to be measured while the arms of the glass Y piece were at a distance from it. At the same time it was possible to make the junction and its wires fit over a straight or curved portion of the skin simply by pulling or relaxing the loose leads at the other end of the Y piece. A very thin wire in close contact with the skin cannot affect the temperature of the underlying skin and it can neither enhance the evaporation of sweat nor interfere with it; heat rays and air currents cannot significantly alter the temperature of the wires without at the same time altering that of the underlying skin; and tests have shown that conduction of heat along that portion of the leads which was inside the glass Y piece did not occur.

The electrode was made up in the following manner. Two thin glass tubes about 10 cm. long were each bent to an angle of 30° about 1·5 cm. from one end, and the tubes were stuck together along their arms by means of sticking plaster. Copper and constantan wires (s.w.g. 36) were soldered together end to end in such a way that the junction was scarcely thicker than the wires and then pulled through the glass tube till the junction lay midway between the open arms of the glass Y piece (Fig. 1). The junction was painted over with a very fine layer of heat-conducting varnish and the wires leading loosely away from the Y piece were encased in polythene tubing. The e.m.f. was read on a portable potentiometer designed for this type of work. The cold junction was embedded in melting ice in an insulated container. The potentiometer was always kept at room temperature and only carried into the cold room or warm room when measurements were made; these never lasted more than a few minutes, and the insulating effect of the box was sufficient to prevent errors caused by temperature changes in the standard cell.

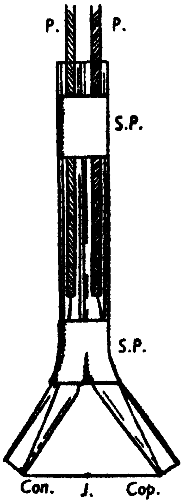

Fig. 1. The thermocouple element, two-thirds natural size. *Con.* = constantan wire; *Cop.* = copper wire; *J.* = junction of wires; *P.* = polythene tubing; *S.P.* = sticking plaster.

Rectal and mouth temperatures. Rectal and mouth temperatures were measured with mercury thermometers over 5 min. to the nearest 0·05° C. The subjects had been taught to insert the rectal thermometers with their right hand through the front flap of their trousers while sitting, and to insert it always to the same distance.

Calibration. The potentiometer was calibrated against three mercury thermometers to the nearest 0·1° C. All mercury thermometers and thermocouples used in this experiment were tested against each other in a stirred water-bath and found to agree to less than 0·1° C. Quick consecutive readings from the same spot of skin agreed to within 0·2° C., even if read by two independent observers. If a thermocouple was held against the sublingual mucosa it gave readings which agreed to the nearest 0·1° C. with those taken by a mercury thermometer.

RESULTS

Only the results obtained from the seven men who took part in the whole experiment are given here.

Cooling

Average results are given in Fig. 2*a*.

Although there were individual variations and day to day differences, cooling generally followed the same pattern. In four out of twenty-eight observations the rectal temperature rose by 0·1–0·4° C. during the first 15 min. Usually it fell slowly at first and more quickly after about 30 min. The average fall of rectal temperature was 0·9° C. during 1½ hr. at 0° C.; (in twenty-three out of twenty-eight observations the fall in rectal temperature was 0·65–1·35° C., but the total scatter was 0·25–1·9° C.). When the mouth temperature was measured it was found to follow the rectal temperature, except that sometimes the former had fallen less than the latter at the end of cooling.

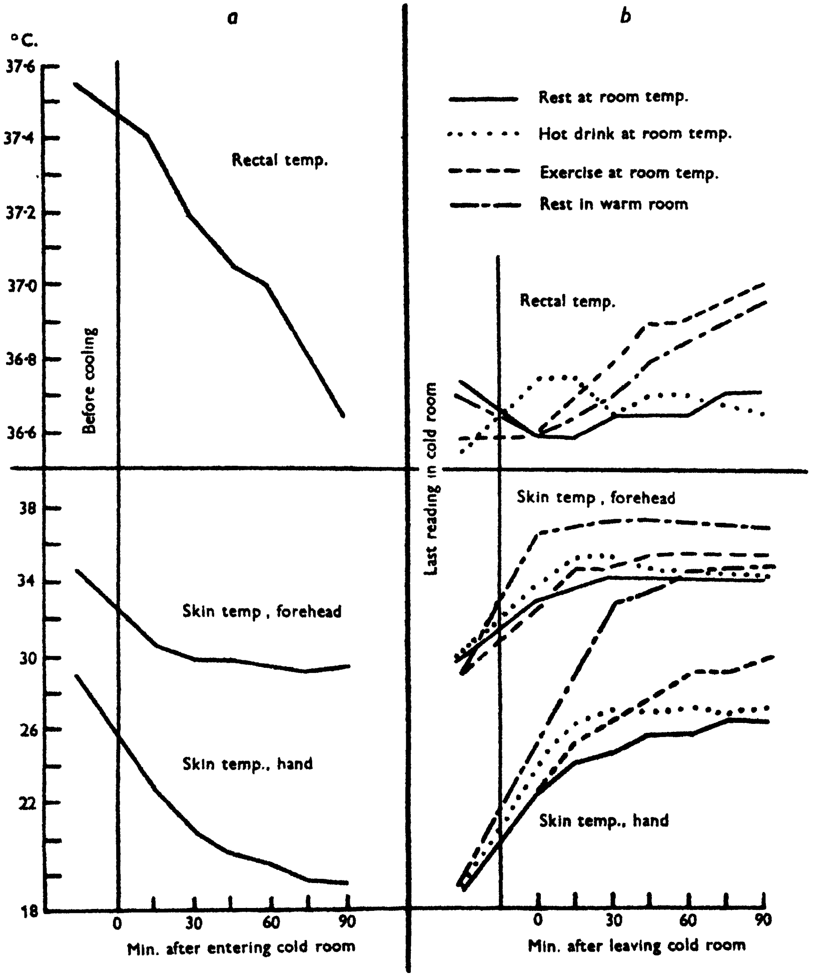

Fig. 2. (*a*) *Cooling* at 0° C. Average of twenty-eight observations on seven subjects. (*b*) *Warming* by different methods. Each line represents the average of seven observations, one on each subject.

The skin temperature of the forehead and hands fell most during the first 15 min. of cooling. In most cases the forehead ceased to cool down after about 30 min. and subsequently its temperature sometimes even rose slightly, but the hand temperature usually continued to fall until the subjects left the cold room.

Average forehead temperatures fell by 4·0° C. (extremes 2·4 and 7·3° C.) in 1¼ hr. and hand temperatures by 11·5° C. (extremes 4·8 and 16·9° C.).

Shivering often began after ¾–1 hr. It did not appear to depend on a fall of the rectal or skin temperature to any particular level, although it was often followed by a slowing down of the fall in rectal temperature or even a slight temporary rise. Some subjects shivered more than others, and some did not noticeably shiver at all on certain days, although their rectal temperature had fallen by up to 1·2° C. and was as low as 35·8° C. Some subjects began to shiver when their rectal temperature was 37·0–37·3° C. and after it had fallen by only about 0·2–0·3° C.

Warming

Average results are given in Fig. 2*b*.

At rest in a hot medium the rectal temperature of one subject was unchanged and that of six subjects had fallen by 0·05–0·35° C. 15 min. after leaving the cold, but it invariably rose later. After 1¾ hr. it equalled the last reading in the cold room in one subject and had risen above it by up to 0·6° C. in the other six; in all subjects, however, it remained 0·45–0·85° C. lower than it had been before cooling.

When exercise was taken at room temperature the rectal temperature of three subjects rose after 15 min. while that of four subjects fell by 0·05–0·35° C. during the first 15 or 30 min. In these again, it invariably rose later, finally surpassing the last reading in the cold room by up to 0·9° C. in five subjects and equalling it in two. It remained, however, 0·45–0·7° C. below the initial level in all subjects.

Following a hot drink at rest at room temperature, the rectal temperature of one subject fell by 0·25° C. and that of the other six rose by up to 0·85° C. during the first 15–30 min. after leaving the cold. Subsequently, however, it fell in all six subjects in whom it had risen first. At the end of 1¾ hr., it was 0·15–0·3° C. below the last reading in the cold room in five of these and 0·45–0·85° C. above it in the other two. In all subjects, furthermore, it remained 0·55–1·05° C. below the initial level.

At rest at room temperature without a hot drink the rectal temperature of one subject showed no change at first, that of one subject rose slightly during the first 15 min. and that of five subjects fell by 0·05–0·7° C. Subsequently, the rectal temperature of all subjects rose, but after 1¼ hr. it was still 0·2–0·4° C. lower than at the end of cooling in three subjects, and 0·45–1·2° C. below the initial level in all subjects.

Although none of the subjects came nearer his initial level of rectal temperature than 0·4° C. in any test, all felt comfortable within at most 30 min. from leaving the cold. Often they had no sensations of cold, even when their rectal temperature was similar to or lower than that at which they had shivered and felt cold before. In all tests the rectal temperature settled down to a steady level about 1–1¼ hr. after leaving the cold, and the greatest fluctuation recorded in any one subject during the last 30 min. of any one test was 0·15° C. Every subject had a higher final level of rectal temperature after exercise and after sitting in a warm room than in the other two tests.

When the mouth temperature was taken it followed roughly the same pattern as the rectal temperature, but the former tended to rise more rapidly and settle nearer its initial level than the latter, especially in the warm room. The mouth temperature rose above the rectal temperature after a hot drink, and it also did so in the warm room (Fig. 3).

The skin temperature of the forehead and hand always rose fastest and highest in the warm room and settled at levels which were distinctly above those of the first observations in the same subject on that day. With the other methods the forehead temperature settled near its initial level after about 30–45 min. At rest at room temperature, with or without a hot drink, the hand temperature settled near or just below its initial level after about 1 hr., but it tended to rise a little longer when exercise was taken and to settle at a slightly higher level. After a hot drink the skin temperature of both points always rose faster, though not higher, than at rest or after exercise at room temperature. During the last 30 min. of exposure to a warmer medium, the skin temperature of the hand and forehead fluctuated by less than 0·6° C. in the majority of tests, and only exceptionally by more than 1·0° C.

On the days when the eighth subject took part in the experiment he produced no results which contradict what has been said above.

Acclimatization

No difference was noticed between the two groups of men or between those who were acclimatized to heat and those who had lived under cold conditions. Day to day changes in cooling did not suggest that acclimatization to cold was achieved during this experiment.

The relationship between mouth, rectal, and skin temperature on warming in a hot medium

As has been stated above, the mouth temperatures rose above the rectal temperature when the subjects went straight from the cold room to the warm room. In all subjects except one, the skin temperature of the forehead also rose above that of the rectum and, when the mouth temperature was recorded, above the latter. Simultaneous readings of the skin temperature of the hand and of random points on the limbs were always below the rectal and mouth temperature. If the thermocouple was held in the air near the forehead a lower temperature was invariably recorded, which shows that these findings were not brought about by direct radiation from the heaters. One of the tests in which the eighth subject took part involved warming in the warm room, and he also showed this phenomenon. Since he was tested under similar conditions in all relevant aspects it seems fair to include him in this particular description. A rise of the forehead temperature above that of the rectum was thus recorded in seven out of eight subjects, and its maximum varied from 0·2 to 1·7° C. A typical record is given in Fig. 3. It was chosen, because it includes a record of the mouth temperature and because the maximum rise of the forehead temperature above that of the rectum equals the average of the maximum rise in all seven observations.

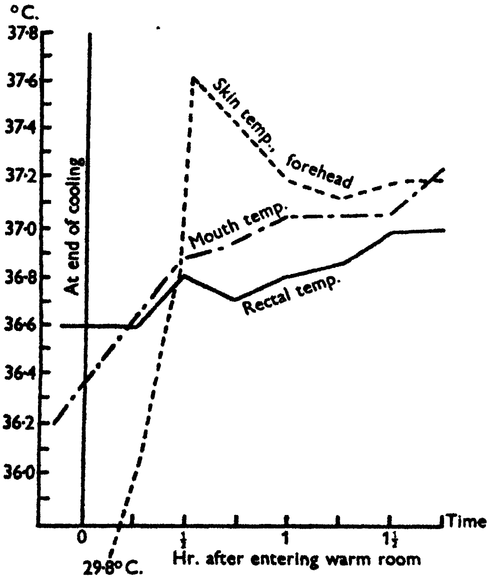

Fig. 3. The relationship between the rectal temperature, the mouth temperature, and the forehead temperature on changing from a cool medium (0° C.) to a warm one (29° C.). Typical record.

Most subjects showed some slight sweating of the forehead about ¾–1 hr. after entering the warm room and then the forehead temperature dropped a little, but it was still above that of the rectum at the end of the test in five subjects. This mild sweating always occurred at rectal temperatures which were lower than the initial level on that day and sometimes also lower than that at which the same subject had begun to shiver on the same day.

DISCUSSION

Shivering. Spealman (1945) found that men in moderately cool baths began to shiver when their rectal temperature had fallen to 36° C. Heat production was increased as a result of shivering, and the rectal temperature remained around 36° C. Spealman thought that 36° C. was a critical level of human heat regulation, but this need not be true outside the carefully standardized conditions of his experiment. Uprus, Gaylor & Carmichael (1935) noted shivering only when the rectal temperature of their subjects was falling, but not when the rectal temperature began to rise again. The present investigation, however, suggests that there is no close relationship between the rectal temperature and shivering. Since there is no evidence that the receptors which initiate shivering lie in the rectum such a relationship appears highly improbable.

The paradoxical behaviour of the rectal temperature at the beginning of cooling and warming. A slight temporary rise of rectal temperature on cooling or a slight temporary fall on warming has been recorded by Barcroft & Verzár (1931), Uprus *et al.* (1936), Alexander (1945), Adolph & Molnar (1946), and Aschoff (1947). Tholozan & Brown-Séquard (1858) noticed that the mouth temperature sometimes rose when a hand was immersed in cold water, while Cooper & Kerslake (1948) noted an initial fall of the mouth temperature on warming human subjects under a cradle. Freeman & Nickerson (1938) actually thought that there was some negative correlation between the temperature of the skin and the rectum. This statement would imply that the rectal temperature is lowest when the skin is warmest and highest when the skin is coolest, but the present investigation and a study of the observations mentioned above suggests nothing more than that the rectal temperature and the skin temperature can sometimes move in opposite directions.

Uprus *et al.* (1936) have briefly touched upon the possible mechanism of such an occasional and temporary rise of rectal temperature when the body is cooled, and this was expanded by Aschoff (1944, 1947). The latter found that dissipation of heat through the hand was greatly reduced when the hand was cooled, sometimes more so than the extent of cooling would warrant, and he thought that such a reduction of heat loss through the hand might at times 'over-compensate' the requirements of the body. This hypothesis may also help to explain why the rectal and mouth temperature occasionally fall on exposing the body surface to heat, for reflex vasodilatation may result in dissipating more heat than necessary, provided the environmental temperature is lower than the skin temperature. In the present investigation this paradoxical fall of the rectal temperature was most consistently noted on entering the warm room when there was a considerable stimulus for superficial vasodilatation.

There appears, however, to be another mechanism. Cooling of the skin results in cooling of the underlying muscles (Bing, Carlsten & Christiansen, 1945) and warming of a limb increases the blood flow through the skin and muscles (Barcroft & Edholm, 1943). Thus, when a chilled person is rapidly warmed, it is probable that a large amount of blood flows through a cold limb. It is known that venous blood in the limbs is usually colder than the blood in the adjoining arteries (Davy, 1814; Bazett, 1947), and Lewis (1927) found that the skin above a vein carrying blood from a hand which had been immersed in cold water was up to 9° C. cooler than the surrounding skin. The rectum, therefore, may be cooled by passage of cool blood from the lower limbs through the haemorrhoidal plexus to the mesenteric vein. Warming of the rectum by passage of warm venous blood through the haemorrhoidal plexus is unlikely to occur, for the limbs are as a rule cooler than the rest of the body, and cooling decreases the blood flow. A reduction of the amount of cool blood flowing through the rectum may, however, result in a rise of the rectal temperature, for the latter would depend both on the quantity and the temperature of the blood flowing through the rectal vessels.

Heat exchanges. Fluctuations of the rectal temperature disappeared after about 1 hr. under constant conditions. Barcroft & Verzár (1931) have suggested that the heat production of a resting man may be insufficient to raise his body temperature after cooling, and a rough estimate of the heat exchanges occurring in the present investigation confirms this assumption. Heat losses of lightly clothed and 'semi-reclining' men were estimated to be 40·5 cal./sq.m./hr. at 29° C. and 76 cal./sq.m./hr. at 17° C. (Gagge, Winslow & Herrington, 1938). At rest in the warm room the subjects of this experiment may thus have given off about 70–75 cal./hr. and at rest at room temperature about 135–140 cal./hr. A generous estimate of their resting heat production would be about 100 cal./hr. The subjects of the present investigation were tested under somewhat similar conditions to those of Gagge *et al.* (1938) and it may be concluded that at rest at room temperature their heat production was lower than their heat losses. It follows that warming of the periphery after leaving the cold room was largely achieved at the expense of cooling other parts of the body, and this explains why in eight out of fourteen tests at rest at room temperature the rectal temperature eventually settled down at a level lower than that at which it had been when the subject left the cold room. The direct warming effect of 1 pint of fluid taken at 45–50° C. was negligible (about 6–9 cal.) and was more than counteracted by the increase of heat elimination which sets in after ingestion of hot fluids (Pickering, 1932). That such an increase of heat elimination did occur in the present experiment is demonstrated by the fact that peripheral vasodilatation after a hot drink was always more sudden than in the other tests at room temperature while the rectal temperature eventually fell below the last reading in the cold room five out of eight times. In the warm room heat losses may have been smaller than heat gains, and eventually some warming of the body as a whole may have occurred. Each 1 min. period of stepping may have raised heat production by about 10 cal. (Lusk, 1928). Under those conditions the subjects may have produced some 40 cal./hr. more than at rest, and their bodies may have again gained heat as a whole.

It can be seen from what has been said above, that the means of warming employed in the present investigation would not normally raise the rectal temperature of healthy men to any significant extent. It might have been expected that people with a lowered overall temperature would have maintained peripheral vasoconstriction until their loss of heat had been made good. In fact, however, their skin temperature rose fast while their rectal temperature remained low, and such a persistent lowering of the rectal temperature could not have been a result of cooling by venous blood. During the last 30 min. of these tests both the skin and rectal temperature remained remarkably steady, even though the forehead was sometimes warmer than the rectum (Fig. 3), which suggests that a balance had been struck between heat production and heat loss at a temperature lower than the initial one. The results published by Freeman & Nickerson (1938), Gagge (1941, table 1), Robinson, Turrell & Gerking (1945), Spealman (1945), and Robinson & Gerking (1947) confirm the view that such a balance can be maintained at various levels of rectal temperature. It appears, furthermore, that *within certain limits* the establishment of thermal balance takes precedence over the temperature of any part of the body (or at any rate the rectum, the mouth and the skin). This view is borne out by a report of Rapaport, Fletcher & Hall (1948), who found that in a cold environment the hand and foot temperature of their subjects could be made primarily dependent on the state of thermal balance of the body and almost independent of the environment. It may be concluded that the methods of warming employed in the present investigation failed to achieve a return of the rectal temperature to its initial level, but that they were sufficient to restore comfort and approximate thermal balance; it does not follow, therefore, that these methods were inadequate.

The meaning of 'adequate warming'. It is evident from what has been said above that a definition of a normal state of body temperature is required, especially under conditions of thermal stress. A definition based on the rectal temperature would be unsatisfactory because, as has been suggested above, heat exchanges can be in equilibrium over a range of rectal temperatures, because the rectal temperature varies from man to man, and because it may be influenced by changes of temperature in the lower limbs. It is necessary, furthermore, to differentiate between a feeling of warmth, depending largely on the skin temperature, and the total amount of heat in the body. In the present investigation the subjects often felt warm after leaving the cold room while their skin temperature was rising and their rectal temperature falling, but such a fall of rectal temperature, coupled with a rise of skin temperature, suggests that the body is losing heat as a whole. One cannot say, therefore, that people are warm when they feel warm, nor can one say that a state of thermal balance represents comfort. The example given above (Spealman, 1945) showed that men might be in thermal balance while shivering with rectal temperatures of about 36·0° C. The table already quoted (Gagge, 1941) shows that naked men were actually giving off a small amount of heat in a hot dry medium when their rectal temperatures averaged 37·56° C., and Robinson & Gerking (1947) have shown that men working in a hot humid medium could be near thermal balance at rectal temperatures around 38·5° C. All these subjects may have felt uncomfortable, and a definition which disregards subjective feelings or the efforts of the body to maintain thermal equilibrium does not seem satisfactory. One must say, therefore, that a satisfactory state of body temperature implies: (*a*) a state of balance or near-balance between heat production, and heat loss, and (*b*) a range of skin temperature which is subjectively comfortable. The latter implies the absence of shivering, sweating or panting.

The mutual relationship of the rectal, mouth and forehead temperature after rapid warming. The finding that the rectal temperature was lower than the mouth and forehead temperature when the subjects were resting in the hot room may have been a result of cold blood flowing through the haemorrhoidal veins (see above), while warm blood from the inner organs was being pumped into the head. The findings, though not the conclusions, of Haldane (1905) suggest that such a flow of warm arterial blood to the head may occur when men are suddenly exposed to a much hotter environment than the one they had been in before. The forehead temperature, however, could have risen above that of the mouth only if there was extreme vasodilatation in the former, and extreme vasoconstriction, coupled with previous cooling, in the latter.

In a recent publication, which was seen after this paper was written, Bazett, Love, Newton, Eisenberg, Day & Forster (1948) suggested that vascular anastomoses may affect the rectal temperature. In another publication Bazett, Mendelson, Love & Libet (1948) described paradoxical changes of the skin temperature during warming after cooling. The latter findings appear to have been associated with the fact that certain superficial blood vessels carried cool blood from the body surface while others carried warmer blood from deeper tissues. This conception expands the tentative explanation put forward in the present paper for the causes of the rise of skin temperature above that of the mouth and rectum.

Practical implications. The relative merits of the various ways of warming any particular person may have to be determined for each and every practical set-up, and they will depend on the extent of previous cooling, the available facilities, and the condition of the subjects. It must be understood that the body as a whole does not immediately gain heat when it moves from a cold medium to a warmer one and that any stimulus causing peripheral vasodilatation may result in loss of heat for it will cause valuable energy to be expended on warming less important organs at the expense of essential ones. A short period in a warm place, therefore, seems undesirable for those who must return to the cold. A hot drink causes an immediate feeling of warmth, but it increases the heat elimination from the periphery, and it appears that its effect is similar to that of alcohol. Both a short period in a warm place and a hot drink, however, may be beneficial when the heat production of the body is adequate and frost-bite is to be forestalled. Voluntary exercise may be beneficial to men who are not shivering. It must be remembered, however, that fatigue predisposes to exhaustion from cold and that shivering alone can cause fatigue (Adolph & Molnar, 1946). It seems safe to conclude that a combination of all three methods, i.e. hot food and drink coupled with exercise in a warm place, would warm up people more rapidly than any one method. This will have to be confirmed by experiment, although the quick effect of a hot drink, the increase of heat production following upon exercise and food, and the lowering of heat losses in a hot place can be expected to have a cumulative effect.

Thermal balance (that is a steady level of rectal and skin temperature) seems to have been established fairly soon in all tests, but this was achieved at a low level of rectal temperature. It is impossible to say without further experimental evidence whether it would be a disadvantage to begin another period of cooling at such a low level, but this possibility must be considered. Animal experiments (O'Connor, 1919, figs. 3 and 5) suggest that heat elimination from a limb was raised for some time after warming of the animal had stopped. It appears, therefore, that men in cold places ought to be kept warm rather than intermittently warmed. Warming by hot water remains the most efficient single method, especially after severe chilling (Alexander, 1945). Even air which is warmer than the skin temperature is less effective than water, because the heat capacity of air is small and because air in the immediate vicinity of the body adjusts itself to the skin temperature (Loewy & Dorno, 1925).

SUMMARY

1. Seven men were moderately chilled and then warmed over 1¾ hr. under the following conditions: (*a*) rest at room temperature, (*b*) a hot drink followed by rest at room temperature, (*c*) alternate periods of rest and exercise at room temperature, (*d*) rest in a warm dry medium.

2. In a warm medium the skin temperature of the forehead and hand always rose above its initial level. In the other tests it settled near its initial level.

3. During warming the rectal temperature always remained 0·4–1·2° C. below its initial level. At rest at room temperature it also tended to settle below the last reading in the cold room, especially if a hot drink was taken.

4. On entering the warm room the forehead temperature of all subjects but one rose by up to 1·7° C. above their rectal temperature. If the mouth temperature was taken at the same time the forehead temperature also rose above the latter.

5. The initial effect of entering a warm room after cooling seems to have been a warming of the surface at the expense of more essential regions. A hot drink appears to have been followed by loss of heat from the body as a whole.

6. It is suggested that venous blood flowing through the haemorrhoidal plexus may cool the rectum.

7. After 1¼ hr. in a warmer medium only insignificant fluctuations of the skin and rectal temperature were recorded. It is concluded that thermal balance was achieved, and it is suggested that, within limits, the establishment of such a balance may take precedence over the maintenance of a constant temperature.

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THE MODE OF REACTION OF INTERSTITIAL CONNECTIVE TISSUE WITH WATER

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When water was injected into the subcutaneous tissues of a freshly killed animal, it was observed to accumulate around the point of injection (Ranvier's 'Boule d'œdème'). Other fluids, such as alcohol, did not localize in this way, but spread evenly and rapidly into the surrounding tissues. These facts suggested a peculiar avidity of the tissue for water. Examination of the swollen tissue with the dark ground microscope showed separation, without visible alteration, of the collagen fibrils. For this reason, it was considered probable that the water had combined with a substance which lay between the fibrils rather than with the fibrils themselves (Day, 1948). A substance of this kind had been previously described (Day, 1947*a*), and Flemming's observation (1876), that, in acid solutions of strength sufficient to cause the collagen fibres to swell, this substance became shrunken, was confirmed.

The present investigation is concerned with those changes in the morphology of interstitial connective tissue which took place, in aqueous solutions, as the result of experimental variation of pH and of neutral salt concentration. The study was undertaken with the object of gaining further knowledge of the mode of reaction of this tissue with water with particular regard to the part played by the interfibrillary substance.

METHODS

The material studied was the deep fascia immediately covering the ventral aspect of the thigh muscles of the rat. Pieces up to 1 cm. wide were removed from a freshly killed animal. Each was placed at once in at least 20 ml. of the fluid in which it was to be studied. The pH of the fluids was measured electrometrically, with a glass electrode apparatus, at the beginning and at the end of the period of immersion of the tissue. The pH of the fluids was varied by the addition of dilute HCl or NaOH. Where solutions of salts were concerned, the quantity of Na^+ or Cl^- so added, was so very small in comparison with the concentrations of the salt ions already present that they could be ignored. Usually, the tissue was left in the fluids overnight and at a room temperature between 18 and 20° C.

The changes which took place were observed with the naked eye and also microscopically using dark ground illumination. A Leitz 'cardioid' type of condenser was employed in conjunction with a Pointolite illuminant.

RESULTS

Changes resulting from variation in pH alone

(*a*) Pieces of fascia were put into weak alkali at pH 11·1. They became at first curled up, white and opaque. Within a few minutes the edges became grey. This process spread inwards so that within an hour each piece had become entirely grey, transparent and greatly swollen.

Dark ground examination showed broadening of the bundles of collagen fibrils, the majority measuring from 10 to 20 μ. across (Pl. 1*b*). The bundles themselves were separated by spaces up to 100 μ. wide. Their constituent fibrils were also separated and, for the most part, quite distinct from each other. They were not swollen.

Pieces were then transferred to weak acid at pH 4·3. Within a few minutes, white opaque areas appeared in them. After half an hour, each was entirely opaque and also markedly shrunken. All parts of it clung together and resisted disintegration by teasing. Microscopically, it was seen that the collagen bundles had become knotted together and superimposed to such an extent that no spaces could be observed between them. The bundles were greatly narrowed (mostly less than 5 μ. wide) and remarkably crimped and coiled up. It was impossible to distinguish individual fibrils within the condensed bundles.

By placing pieces of tissue alternately in weak acid and alkali, within the limits of pH 4·5–11·0, the processes of shrinkage and swelling were repeated several times. But after 24 hr. the tissue responded more slowly and the effects were less pronounced.

(*b*) Pieces of fascia were treated overnight at a series of pH values ranging from 3·0 to 12·0. The initial and final pH value of the fluids is recorded in the order given. The changes which took place were as follows:

pH 3·8–3·8. Tissue transparent and swollen. Microscopically, considerable swelling of the collagen bundles up to 50 μ. in width with complete disappearance of outline of their constituent fibrils.

pH 4·5–4·7. Tissue white, opaque, very shrunken and hard. Microscopically, collagen bundles narrowed to less than 5 μ. wide, very crimped and knotted together. No spaces between them other than those produced artificially by teasing.

pH 5·0–5·7. Tissue opaque, grey-white, and slightly shrunken. Microscopically, collagen bundles narrow and condensed to less than 5 μ. wide, but well separated from each other (Pl. 1*a*).

pH 7·6–7·3. Tissue grey-white and semi-opaque. Microscopically, collagen bundles mostly from 10 to 5 μ. wide. Their individual fibrils somewhat compressed but distinguishable from each other. Bundles well separated by apparently empty spaces.

pH 11·2–10·9. Tissue grey and transparent. Microscopically, collagen bundles mostly from 20 to 10 μ. wide, their constituent fibres being definitely distinguishable and in some bundles exhibiting a continuous shimmering vibration. Bundles themselves also well separated (Pl. 1*b*).

pH 11·7–11·5. Tissue transparent and swollen. Microscopically, considerable swelling of collagen bundles, with swelling and disappearance of outline of their constituent fibrils.

It is to be concluded that variation in external pH between 4·5 and 11·0 was accompanied by changes in the volume of the tissue to which neither the fibres nor the cells appeared to contribute. Increase in acidity caused shrinkage, and decrease caused swelling of the tissue. Swelling was accompanied by separation of the collagen bundles and fibrils. With shrinkage these structures became drawn together. These changes were reversible. Swelling which occurred at pH values immediately outside this range was of a different character, inasmuch as it manifestly involved the collagen fibrils themselves.

Changes resulting from variation in neutral salt concentration alone

Pieces of fascia were treated overnight in approximately neutral solutions of NaCl at the following concentrations: 4·0, 2·0, 1·0, 0·5, 0·25, 0·125, 0·1, 0·05, 0·025, 0·012 M. One further piece was left in neutral distilled water. The pH of all the fluids used, measured before and after these periods of immersion of the fascia, lay between 8·3 and 6·2. The majority of the values were closer to neutrality. The pieces were examined and then transferred to neutral distilled water for a further period of 18 hr. They were then examined again.

It was found that the change which took place in concentrations of NaCl between 2·0 and 0·125 M was identical with that which occurred in weak alkali. The tissue became swollen and this swelling was accompanied by separation, without increase in size, of collagen fibrils. The change was reversed by treatment with neutral distilled water.

The concentration of NaCl in which the change began to take place was critical. It was observed in a concentration of 0·125 M but not in one of 0·1 M. The pieces which had been treated in solutions of 0·1 M strength and under did not differ in either naked eye or microscopic appearance from that which had been treated with neutral distilled water alone.

The change which took place in a 4·0 M solution was not completely reversible and differed in other respects from that which was seen in the lower concentrations.

Changes resulting from variation in both neutral salt concentration and pH

The following concentrations of NaCl were prepared: 1·0, 0·5, 0·25, 0·125, 0·062 M. Six test-tubes were filled with solution at each of these concentrations. Six further tubes were filled with distilled water. The fluid in one out of each group of six tubes was adjusted as nearly as possible to one of the following pH values: 3·0, 4·0, 5·0, 7·0, 11·0 and 12·0. The precise pH value of the fluid in each of the forty-two tubes was then measured and a piece of fascia inserted. The tubes were left overnight at a temperature of between 18 and 20° C. The pH value of the fluids was then measured again. The naked eye and microscopic appearance of each piece was then examined.

The results of this experiment are recorded in Table 1. The appearances of the tissue are represented by the letters O, T, SO and C. O, T and SO indicate naked eye changes which contrasted with each other to an extent which quite excluded subjective error. Microscopically these changes were seen to be accompanied by alteration in the spatial relationships of the collagen fibrils to each other, but by no visible change in the fibrils themselves. The letter C, on the other hand, indicates swelling of the tissue accompanied by visible swelling of collagen fibrils.

TABLE 1. Changes in rat fascia after immersion in aqueous solutions of sodium chloride of varied strength and at different pH values

NaCl concentration	Approx. pH					
	3	4	5	7	11	12
M	O	O	T	T	T	C
M/2	O	O	T	T	T	C
M/4	C	O	O	T	T	C
M/8	C	O	O	T	T	C
M/16	C	O	O	SO	T	C
Water	C	O	O	SO	T	C

O = Opacity and shrinkage. T = Transparency and swelling. SO = Semi-opacity without notable change in size. C = Swelling of collagen fibres. Time of immersion = 18 hr. Temperature, 20–18° C.

From the evidence summarized in Table 1 two main conclusions were drawn: (1) That at pH values in the region of 4·0, salt, even in molar concentration, had no swelling action on the tissue. (2) That with variation of pH from 4·0 to 11·0, the concentration of salt necessary to cause swelling became proportionately less.

Comparison of the effects of different neutral salts

It has been mentioned both here and previously (Day, 1947*a*) that, in certain circumstances, collagen fibrils may exhibit a continuous shimmering vibration. The vibration has been observed to continue for several days in the same preparation and is in the nature of Brownian movement. It was only seen in tissue which had become swollen and appeared to be related to the degree of separation of the fibrils.

It was found that, under standard conditions of temperature, illumination, and pH, the lowest concentration in which Brownian movement of collagen fibrils could just be detected, was always the same for each particular salt. If it is assumed that the occurrence of this phenomenon is a measure of a certain degree of interfibrillary hydration of the tissue, assessment of this minimal concentration, under standard conditions, may be expected to provide a means of comparing the hydrating capacity of each of a series of salts.

It is to be emphasized, however, that the swelling effect of salts on the tissue can be seen with the naked eye. Where, as in the case of NaCl, and $Na_3C_6H_5O_7$, the salts differ greatly in their hydrating capacity, this difference may easily be observed by mere naked eye inspection. The value of Brownian movement, as a criterion of a precise degree of hydration, was mainly in the comparison of salts between which the difference in hydrating capacity was slight.

TABLE 2. The lowest concentrations of neutral salts in which Brownian movement of the collagen fibres was observed compared with the nature of their ions

Salt	pH value of solution at beginning and end of period of immersion of tissue	Lowest dilution in which Brownian movement of collagen fibres was observed	Valency of anion
NaCl	9·86–9·30	M/7	1
Na_2SO_4	9·46–8·70	M/16	2
$Na_3C_6H_5O_7$	8·32–8·12	M/25	3
			Length of crystal radius of anion (A)
NaF	6·42–6·54	M/3	1·36
NaCl	6·20–6·20	M/6	1·81
NaBr	6·30–6·30	M/7	1·95
NaI	6·08–6·07	M/8	2·16
			Length of crystal radius of kation
LiCl	6·40–6·90	M/4	0·60
NaCl	6·20–6·20	M/6	0·95
KCl	5·84–5·84	M/7	1·33
RbCl	5·60–5·54	M/8	1·48

Time of immersion, – 3½ hr. Temperature 20° C.

The salts were compared in groups, each group being investigated on the same day. The pH values of the dilutions of salts in each group were adjusted, as nearly as possible, to the same figure by the addition of weak HCl or NaOH diluted in a solution of the appropriate salt. The experiments were carried out at room temperature adjusted to 20° C. Pieces were left for at least 3½ hr. It was found that Brownian movement could be seen best in bundles which had been dissociated from the main mass of the tissue. After removal from the tube, each piece was, therefore, gently teased before being mounted under a cover-slip in a drop of the solution in which it had been lying. The precise pH values of the solutions were measured at the beginning and at the end of the period of immersion of the tissue. Each preparation was examined as soon as it had been made, using a $\frac{1}{12}$ objective and a × 10 eyepiece. The same source of light was used throughout and was adjusted to maximum intensity for each observation.

The results of this experiment are recorded in Table 2.

The effect of neutral salts compared with that of urea and of glycine

Pieces were placed in 1·0, 0·5, 0·25, 0·125, 0·062 M solutions of urea. They were left for 6 hr. at a temperature of 20° C. The pH values of the solutions ranged between pH 5·4 and 6·8. No appearance was observed in any of the pieces other than would be expected, between these pH values, in water alone.

Pieces of fascia were treated over the same period with the same range of concentration of glycine. The pH values of the solutions remained at pH 6·0 throughout the period of the experiment. No change in the tissue was observed which could be attributed to the presence of glycine.

DISCUSSION

The swelling and shrinkage which takes place in fascial connective tissue as the result of variation in external salt concentration and of pH between the values 4·5 and 11·0, has been shown to be related to the degree of separation of the collagen fibrils. It is probable, therefore, that these changes occur in the interfibrillary substance. Other possibilities must, however, be considered.

In the first place the changes may be due, at least in part, to alteration of the fibrils themselves, a possibility suggested by the extreme crimping which they were seen to undergo around pH 4·5–5·0. Against this is the observation (Day, 1947 *a*) that isolated fibrils from tendon in a rat's tail, which are not associated with interfibrillary substance, do not crimp in weak acid. It might be argued that the fibrils of tendon and fascia have a different composition. However, both types of fibril become swollen in approximately the same minimal strengths of acid or alkali and their staining reactions and appearance in stained sections are similar. It would seem unlikely, therefore, that the fibrils of fascia and tendon differ sufficiently in composition as to explain this marked contrast in behaviour.

A further possibility is that the fibrils are not joined together into bundles by an interfibrillary substance but owe their cohesion entirely to physico-chemical forces. On this supposition, the separation of the fibrils which has been observed at certain ionic concentrations is due to weakening or abolition of these cohesive forces. Such a process would, however, be expected to result finally in the complete separation of the fibrils and in permanent disorganization of their arrangement in bundles. It has been shown that this result is not characteristic of the action of either neutral salts or weak alkali. Even in tissue which has become very greatly swollen, the fibrils still remained arranged in bundles and maintained a fixed relationship to each other: furthermore, the change was reversible so that with removal of the external conditions which determined swelling, the fibrils in each bundle came together again.

The action of trypsin, which digests the interfibrillary substance, leads to quite different effects. Individual fibrils then become entirely dissociated from each other and the spaces between fibrils can no longer be made to vary in size with change in ionic environment. Moreover, the change produced by trypsin is irreversible and leads to permanent disorganization of the tissue.

A point of some interest is that tryptic digestion of the interfibrillary substance does not lead to the appearance of Brownian movement of the fibrils, even when transferred to conditions which would have brought about this phenomenon in fresh tissue. Fibrils which become completely isolated and which lie suspended free in the fluid, occasionally show a sluggish movement but the vigorous shimmering vibration described above was not seen. It would appear that this vibration is not a property of the fibrils themselves but depends upon their association with the interfibrillary substance.

On the whole, there would seem to be good grounds to suppose that the changes in rat fascia described above, are mainly due to the presence in it of an interfibrillary substance. It is proposed, therefore, to consider the nature of this substance more fully.

The nature of the interfibrillary substance. In spite of its hydrophilic character, it is impossible to leach the substance from the tissue by prolonged perfusion with water (Day, 1948). Above, it has also been shown that the extreme degrees of swelling and shrinkage, which have been produced experimentally, are reversible and could be repeated several times without any disorganization of the tissue detectable with the dark ground microscope. Extreme hydration of the substance does not, therefore, lead to its solution. In view of the obviously colloidal nature of the substance, this fact can best be explained by assuming that the non-aqueous component of the colloid must exist as a continuum rather than as separate particles.

The structured nature of the substance may also be inferred from histological evidence. In certain circumstances it may be altered in such a way as to be easily visible with the dark ground microscope. It then appears in the form of membranes of extreme thinness containing very delicate, branching fibrils (Day, 1947*b*).

The assumption, that the substance consists of molecules held together in a continuum, also implies the existence in it of chemical linkages of a kind compatible with polymerization. It is of considerable interest, therefore, that it can be digested by trypsin (Day, 1947*a*), and that the contraction of the substance which followed treatment either with 60% ethyl alcohol or by adjustment of external pH to values around 4·0, greatly accelerated the digestion (Day, 1948). Since trypsin specifically attacks peptide linkages, these observations strongly suggest that it is by these that the molecules of the continuum are joined together.

It was observed that, over the entire range studied, the interfibrillary substance responded by change in volume to slight variations in external pH. For this reason the microscopic appearance of the tissue was quite typical for any particular pH value. This would imply that its structure may be modified by very small forces. In contrast with fibres, therefore, its internal scaffolding must be considered to be weak. The fact that the substance may readily be transformed into visible membranes suggests that the continuum of molecules, which has been postulated, may be arranged two-dimensionally: an arrangement to which an extensible trellis would seem to provide a suitable analogy. It is clear that a two-dimensional structure of this kind would not afford such good opportunity for the formation of intermolecular linkages as does the linear arrangement of molecules in a fibre. It is to be concluded that the interfibrillary substance belongs essentially to the class of structured proteins. But this conclusion does not exclude the possibility that molecules of a non-protein nature may be articulated in some fashion to the main framework.

The non-participation of collagen fibrils in the changes described. It has been shown that pieces of fascia underwent considerable changes in volume when immersed in aqueous solutions of varied pH and salt content. In all concentrations of salt, and at pH values between 4·5 and 11·0, these changes could not be accounted for by variation in the size of the collagen fibrils. With slight variation of pH beyond this range, the swelling of the fibrils was, however, obvious and practically maximal.

The forces which lead to the swelling of a collagen fibril arise as the result of ionization of its protein molecules (Lloyd & Shore, 1938, ch. XI) and are therefore proportional to variation in pH, relative to the isoelectric point of collagen. The fact that swelling can only take place at pH values outside a certain range suggests that, within this range, the forces which cause swelling are counterbalanced by those of cohesion.

Astbury (1933, pp. 35-6) has vividly described the way in which cohesion between molecules increases in proportion to their length. Collagen fibres are built up of long molecules arranged in a more or less parallel fashion (Lloyd, 1941). This arrangement not only facilitates the formation of lateral linkages between adjacent molecules but also enormously increases their cohesive effect. Even as weak a link as the hydrogen bond is considered to contribute very greatly to the structural stability of a collagen fibre on account of the parallel orientation of the long molecules between which it develops Lloyd, 1941, p. 282). On purely theoretical grounds, therefore, the internal scaffolding of a collagen fibril is likely to be strong and it is to its strength that the observed non-participation of collagen fibrils in swelling changes must largely be due.

The essentially colloidal nature of interstitial connective tissue has been emphasized by Schade (1913, 1927) and by Schade & Menschel (1922). These workers recognized that the cells in this tissue were too sparse to contribute significantly to the great degree of swelling of which it is capable. They considered that swelling was due to physico-chemical changes in an extracellular 'colloid mass' (Schade, 1927) consisting of fibres and an undifferentiated ground substance. Swelling of the fibres and of the ground substance was determined by precisely opposite conditions. This finding led to the conclusion that the water content of connective tissue was controlled by a swelling antagonism—'Quellungsantagonismus'—between these two main colloidal components.

This conclusion is not supported by the observations which have been recorded here. The ranges of pH and of salt concentration in which no swelling of collagen fibrils was observed both included and exceeded such concentrations as are likely to be present in living tissue. It is clear, therefore, that as far as the living animal is concerned, Schade's thesis of a swelling antagonism between fibres and interfibrillary substance cannot be sustained.

Changes in the substance due to variation in pH. It was observed that fresh pieces of fascia, when put into different aqueous solutions, immediately contracted. This contraction occurred irrespectively of the composition of the solutions and must be presumed to be due to the retraction of elastic fibres. In alkaline solutions this contraction was gradually overcome so that in about half an hour the pieces were greatly swollen. In acid, on the other hand, the contraction was intensified to such an extent that dissociation of the tissue with a needle was almost impossible. These observations suggest that the swelling and contraction of the tissue which results from change in external pH is accompanied by the development of considerable mechanical force. It is proposed to consider how such forces may be generated.

It has been concluded that the interfibrillary substance is essentially a continuum of protein molecules. It has been shown (Day, 1947*a*) that it became maximally constricted at external pH values between 4·2 and 3·8. It is therefore probable that the isoelectric point of these molecules lies between these values. The influence of variation in pH upon the hydration of a system of this kind has been treated theoretically by Lloyd & Phillips (1933). Thus (p. 137) 'The molecules of an organized protein can approach as closely as the length of their side chains permits. At the isoelectric point, when each molecule of the structure contains anionic and kationic centres, they will tend to be drawn together by electrostatic attraction which will favour the closest degree of packing and hence the lowest degree of hydration. On either side of the isoelectric point each protein molecule contains predominantly either anionic or kationic centres and the molecules mutually repel each other. By this means, the spaces between the molecules in an organized protein are increased, leading to an increase in the amount of water which can enter freely between the individual molecules.'

Swelling, however, is assisted by other factors. Repulsion of like charges must lead to isolated charged centres around which, on account of their dipolar nature, water molecules will become oriented. Furthermore, the outer surface of the piece of tissue, as its swells, must be considered as a semipermeable membrane, since the protein molecules, being joined together, cannot pass beyond it. This the diffusible ions, which increase in number proportionately as the external pH is removed from the isoelectric point, are certainly able to do. In accordance with the theory first elaborated in relation to the swelling of gelatin by Procter (Lloyd & Shore, 1938, ch. XI) the resulting electrostatic inequality must lead to the further indrawing of water into the system.

Fundamentally, therefore, the forces which lead to swelling and shrinkage of the tissue are electrostatic: they are generated as the result of ionization or suppression of ionization of the multipolar molecules of which the interfibrillary substance must be considered to be composed.

Swelling caused by neutral salts. It has been shown that the non-electrolyte urea and the dipolar ion glycine did not cause the tissue to swell. This fact alone suggests that the swelling effect of neutral salts is due to their existence as free ions. In 1905 J. Mellanby wrote: 'Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of their valencies.' It is probable that it is to precisely the same interionic forces which Mellanby has shown to cause solution of globulin, that the hydration of the interfibrillary substance is due.

The minimal concentration of NaCl necessary to cause visible swelling was found to be critical. It is probable that here, as in the case of the swelling of collagen fibrils, visible change can only occur when the forces which promote swelling have overcome those of internal cohesion; that is to say when the strength of the forces exerted by the free ions in the solution is sufficient. The degree of cohesion within the interfibrillary substance has been shown to vary sensitively with external pH. The observed relationship between the concentration of NaCl necessary to cause swelling and the prevailing pH (Table 1) is therefore to be expected.

Comparison of the capacities of NaCl, Na_2SO_4 and $Na_3C_6H_5O_7$, to bring about the same degree of interfibrillary hydration (Table 2), revealed a relationship between the effective concentration of each salt and the valency of its anion. Although the minimal effective concentrations of these salts differed considerably (0·14, 0·06, 0·04 M), in the order given, the ionic strengths of these concentrations, as calculated from $\mu = \Sigma \frac{1}{2} c_1 v_1^2$ (where μ is the ionic strength, c_1 the actual concentration of each ion, and v_1 its valency), lay much closer together (0·14, 0·18, 0·24).

The observed relationship between the effective concentration of a salt and the valency of its anion would suggest, therefore, that the capacity of a neutral salt solution to cause interfibrillary hydration is determined fundamentally by its ionic strength.

Comparison of the effects of the chlorides of alkali metals and of the halides of sodium, showed that differences in capacity to promote swelling existed between univalent ions of the same sign. These differences appeared to be related to the position of the ions in the lyotropic series (Lloyd & Shore, 1938, p. 405), and may therefore indicate a competition for water between the salt ions and the interfibrillary substance: a competition which, in higher concentration, would result in 'salting out'. But, as has been shown in Table 2, there would also seem to be a relationship between the capacity of ions to hydrate the substance and the length of their radii. Since this is a factor which to some extent determines the strength of interionic forces (Lloyd & Shore, 1938, p. 412), the observed differences in the hydrating capacity of these salts may not be due entirely to differences in lyotropism.

Study of the effect of pH upon the interfibrillary substance has been shown to be fundamental to the elucidation of its structure and mode of hydration. In the living body, however, it is to be expected that variation in pH must play an insignificant role in determining the condition of connective tissue—since living cells could not survive at pH values such as have been shown to cause changes in the tissue *in vitro*. This is not so of salt. Acting at pH values approximating to that of the blood, the lowest concentration of NaCl which occasioned visible change in the tissue was 0·125 M (0·73 %). With increase of concentration to 0·25 M (1·46 %) the hydration of the tissue was very greatly increased. It is of considerable significance that these values lie on either side of that concentration of NaCl which is isotonic with the cells of the body.

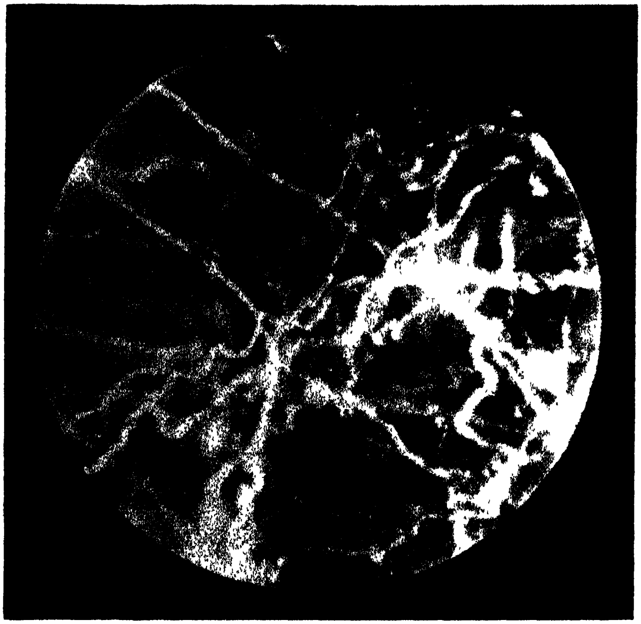
SUMMARY

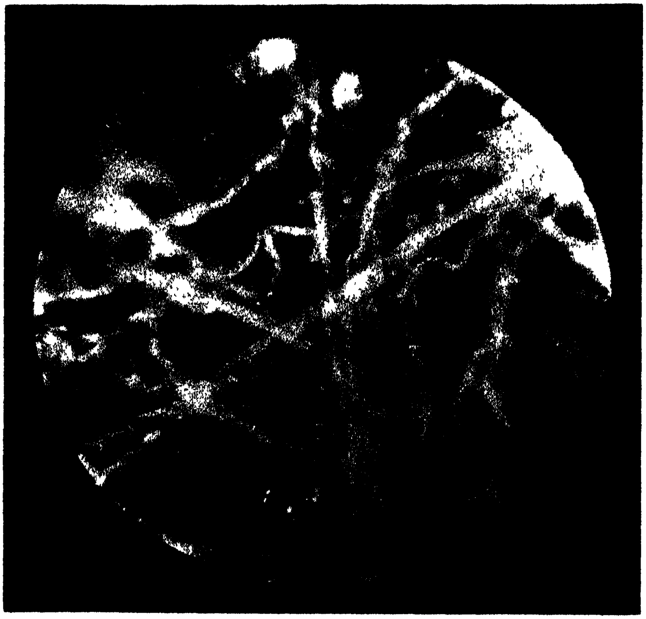
1. Changes which take place in rat interstitial connective tissue, as the result of variation in external pH between 4·5 and 11·0 and in up to molar concentrations of neutral salts, occur exclusively in the interfibrillary substance. The fibres and cells of the tissue take no significant part.

2. The interfibrillary substance is a hydrophilic colloid having a continuous non-aqueous component held together by linkages which are disintegrated by trypsin. It therefore belongs to the category of organized protein. The isoelectric point of this protein lies between pH 4·2 and 3·8.

3. The internal scaffolding of the substance is weak and yields to small forces. It is for this reason that its hydration is modified by very slight changes in external pH and that changes occur in it in circumstances in which collagen fibres remain unaltered.

4. Variation in the hydration of the substance under the influence of change in external pH is considered to be due to attraction or repulsion of charged centres which develop as the result of ionization or suppression of ionization of its multipolar protein molecules.





b

5. The hydrating action of neutral salts on the substance is due to forces set up by their free ions. In the living animal this action of neutral salts is probably of greater importance than that of pH.

6. Schade's conception of a 'swelling antagonism' between fibres and interfibrillary substance is criticized in the light of these findings.

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EXPLANATION OF PLATE

a. Dark ground microscopic appearance of fascia which has come to equilibrium with an external pH of 5·7 The collagen bundles are compact and their constituent fibres cannot be identified. With further acidification to pH 4·5, the bundles would themselves be drawn together, to such an extent that the entire microscopic field would become opaque and unsuitable for photography. × 360.

b. Dark ground microscopic appearance of fascia at equilibrium with an external pH of 11·0. The bundles are widened and the individual fibrils are separated from each other and can be identified individually. × 360.

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EFFECTS OF ELECTRICAL STIMULATION OF THE AORTIC NERVE ON BLOOD PRESSURE AND RESPIRATION IN CATS AND RABBITS UNDER CHLORALOSE AND NEMBUTAL ANAESTHESIA

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The effects of rectangular wave electrical stimulation of the carotid sinus nerve in cats, in rabbits and in dogs have been reported in previous papers (Neil, Redwood & Schweitzer, 1948, 1949*c*, *d*). It was found that the effect of such stimulation upon the arterial blood pressure was dependent upon the type of anaesthetic employed and on the pulse duration of the stimuli used. Thus, in cats under nembutal (pentobarbital sodium) or chloral hydrate anaesthesia, or, in decerebrate cats, stimulation of the carotid sinus nerve, caused a fall in blood pressure irrespective of the pulse duration of the stimuli used. Under chloralose anaesthesia, however, stimuli of short duration (0·05–0·1 msec.) invariably produced a marked pressor response, whereas longer pulses (0·5–1·0 msec.) occasionally lowered the blood pressure. It was also found that intravenous injection of 0·05 g. or more of chloralose into cats under nembutal anaesthesia or in decerebrate cats, converted the fall in blood pressure, consequent on stimulation of the carotid sinus nerve, into a rise.

Analysis of these phenomena showed that chloralose diminished the sensitivity of the carotid baroceptors and also reduced the vasomotor centre response to stimulation of the carotid sinus nerve fibres. The rise in blood pressure caused by stimulation of the carotid sinus nerve in cats under chloralose anaesthesia was thought to be due to excitation of the chemoceptor afferent fibres contained in the nerve trunk.

Similar experiments showed that rectangular wave stimulation of the carotid sinus nerve in rabbits and in dogs caused a fall in arterial blood pressure, irrespective of the anaesthetic used or of the pulse duration of the stimuli employed.

It is generally held that the carotid sinus nerves and aortic nerves form a functional entity. Consequently, the effects of stimulation of the aortic nerves have been examined in cats and in rabbits. A short summary of the results of these experiments has previously been presented (Neil, Redwood & Schweitzer, 1949*a*).

METHODS

Cats and rabbits were anaesthetized with chloralose (0·08–0·1 g./kg. body weight, intravenously) or nembutal (35–40 mg./kg. body weight, intraperitoneally).

The aortic nerves were isolated in the neck at the site of their junction with the superior laryngeal nerve. The nerves were stimulated by means of fine shielded silver wire electrodes (mounted in perspex). A rectangular wave electronic stimulator which allowed independent variations of frequency of stimulation (1·5–1000 cyc./sec.), pulse duration (0·02–10·0 msec.) and intensity (0–100 V.) was used.

Blood pressure was recorded from the femoral artery. A tracheal cannula was inserted. Respiration was recorded by a stethograph or from changes in intratracheal pressure.

In some animals left intraventricular or intra-aortic cannulation was effected (Comroe, 1939). Injections of nicotine (0·04–0·10 mg.) or α-lobeline (0·3 mg.) were given through the cannula in order to stimulate the chemoceptors of the aortic body. Intraventricular position of the cannula was verified by post-mortem examination.

RESULTS

Effects of stimulation of the aortic nerves in rabbits

Stimulation of either the left or right aortic nerve in the rabbit with constant frequency (50 cyc./sec.) of stimulation produced a fall in arterial blood pressure, which varied directly with the pulse duration (Fig. 1). Increasing the frequency of stimulation, with pulse duration constant (1 msec.), increased the hypotensive effects (Fig. 2).

The anaesthetic used did not affect the type of response to aortic nerve stimulation. The tracings for Fig. 1 were obtained under chloralose anaesthesia; those for Fig. 2 were obtained under nembutal anaesthesia.

It was impossible to convert the fall of arterial blood pressure, caused by stimulation of the aortic nerve in rabbits under nembutal anaesthesia, into a rise by repeating the stimulation after intravenous injection of chloralose (Fig. 3*A*, *B*). These results are similar to those found in experiments in which the carotid sinus nerve in rabbits was stimulated (Neil *et al.* 1949*d*).

Effects of stimulation of the aortic nerves in cats and modifications caused by the anaesthetic

'Nembutal' anaesthesia. Stimulation of the left or right aortic nerves caused a fall in the arterial blood pressure, influenced only in magnitude by variation of the pulse duration. A typical response to stimulation is shown in Fig. 4. Only if the animal was heavily overdosed with nembutal (e.g. 100 mg./kg., intraperitoneally) was a pressor response obtained (cf. Douglas, Innes & Kosterlitz, 1948).

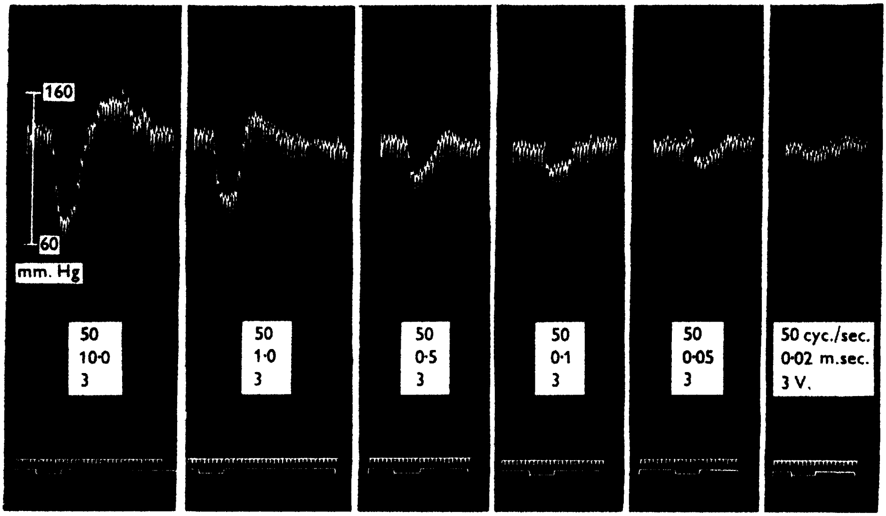

Fig. 1. Rabbit, chloralose anaesthesia. Records from above downwards: blood pressure, time in 5 sec., signal. Stimulation of right aortic nerve with 50 cyc./sec., 3 V., and decreasing pulse durations (10·0–0·02 msec.).

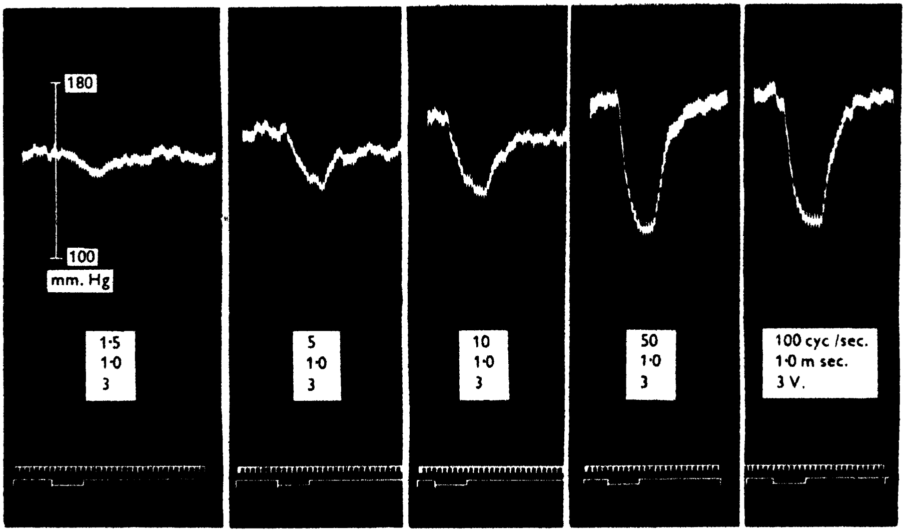

Fig. 2. Rabbit, nembutal anaesthesia. Records as in Fig. 1. Stimulation of left aortic nerve with 1·0 msec., 3 V., and increasing frequencies of stimulation (1·5–100 cyc./sec.).

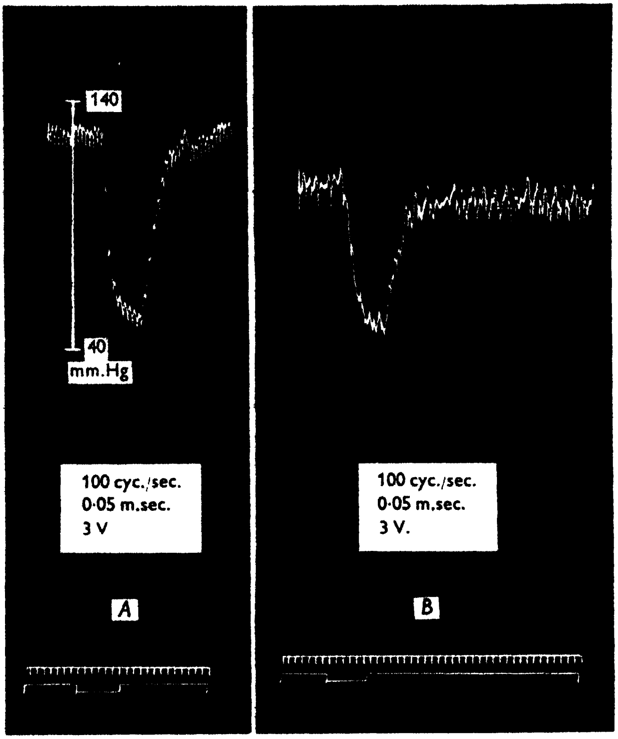

Fig. 3. Rabbit, nembutal anaesthesia, followed by chloralose. Records as in Fig. 1. Stimulation of left aortic nerve with 100 cyc./sec., 0·05 msec., 3 V. *A*, before; *B*, 15 min. after, intravenous injection of chloralose (0·07 g./kg.).

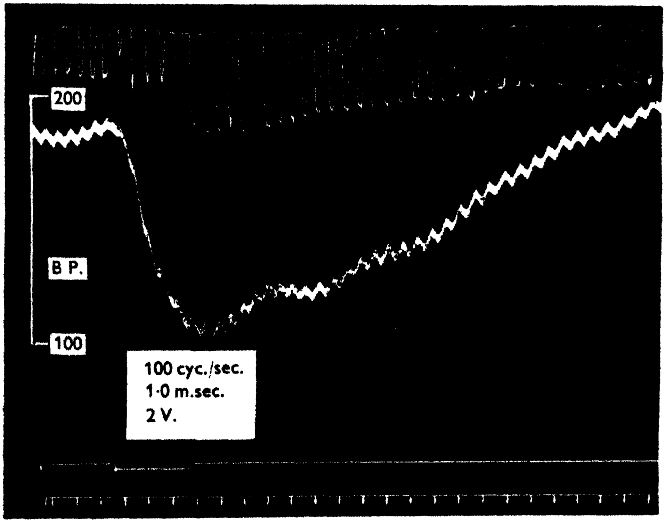

Fig. 4. Cat, nembutal anaesthesia. Records from above downwards: respiration, blood pressure, signal, time in 5 sec. Stimulation of left aortic nerve (2 V., 100 cyc./sec., 1 msec.).

Chloralose anaesthesia. Stimulation of the left aortic nerve in cats produced effects on the arterial blood pressure which were dependent on the pulse duration of the stimuli used. Pulses of 0·1–1·0 msec. duration caused a rise in blood pressure, usually attended by hyperpnoea, whereas pulses of 10·0 msec. duration caused a fall in blood pressure, accompanied by variable effects on respiration (Fig. 5). These results are similar to those obtained by stimulation of the carotid sinus nerve in cats under comparable experimental conditions (Neil *et al.* 1948, 1949*c*). It must be added, however, that the actual range of pulse durations of the stimuli producing these effects was different for the two on nerves. Thus, whereas pulses of 0·02–0·1 msec. duration caused pressor effects, stimulation of the carotid sinus nerves, pulses of 0·5–1·0 msec. duration, were sufficient to produce a fall in arterial blood pressure. In each case the stimulation intensity lay in the range of 1–3 V. Stimulation of the right aortic nerve in cats under chloralose anaesthesia usually produced a marked rise in arterial blood pressure, irrespective of the pulse duration or of the initial level of blood pressure (Fig. 6).

Decerebrate cats. Stimulation of the left aortic nerve in decerebrate cats produced a marked fall in arterial blood pressure, using stimuli of 100 cyc./sec. and 1·0 msec. duration. Intravenous injection of 0·1 g./kg. body weight of chloralose converted this depressor effect into a marked rise within 10–20 min. (Fig. 7). This finding again is similar to our previous observations on the effects of chloralose on the blood-pressure response to carotid sinus nerve stimulation in decerebrate cats (Neil *et al.* 1948, 1949*c*).

Chloralose following nembutal anaesthesia. Fig. 8 shows a typical experiment on a cat. Fig. 8*A* shows the response of the arterial blood pressure to stimulation of the left aortic nerve. Fig. 8*B* was obtained 30 min. after intravenous injection of 0·05 g. of chloralose. Stimulation of the aortic nerve now causes a pressor response. Again the results are similar to those obtained with the carotid sinus nerve. In this latter case, the present authors suggested that pressor effects caused by stimulation of the sinus nerve trunk were due to excitation of the chemoceptor fibre component. The following experiments were performed to show whether the aortic nerves contain fibres from the aortic chemoceptors.

Stimulation of aortic chemoceptors by intraventricular injection of nicotine or lobeline

Comroe (1939) showed in the cat that intra-aortic injection of small doses of nicotine or of lobeline did not produce stimulation of the aortic chemoceptors, but that intraventricular administration of these substances did. He concluded that the arterial supply of the aortic body was derived from the coronary arteries. These findings were confirmed by Gernandt (1946), and our experiments have led to similar conclusions. Hence, in this series of experiments, drug

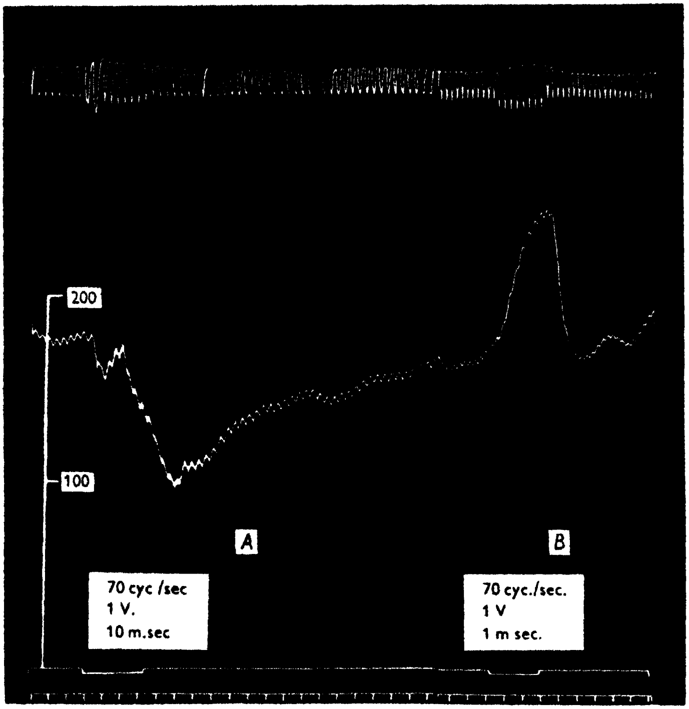

Fig. 5. Cat, chloralose anaesthesia. Records as in Fig. 4. Stimulation of left aortic nerve. Stimulation of 1 V., 70 cyc./sec. *A*, with pulse duration of 10 msec.; *B*, pulse duration of 1 msec.

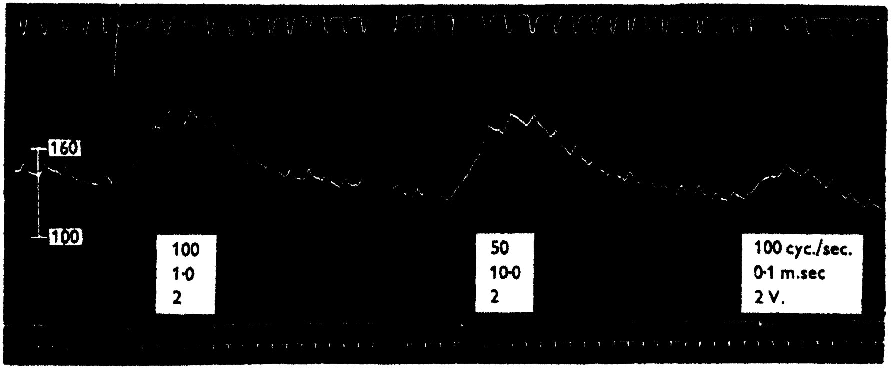

Fig. 6. Cat, chloralose anesthesia. Records as in Fig. 4. Stimulation of right aortic nerve, 2 V. 100 cyc./sec. 1 msec.; 50 cyc./sec., 10 msec.; 100 cyc./sec., 0·1 msec.

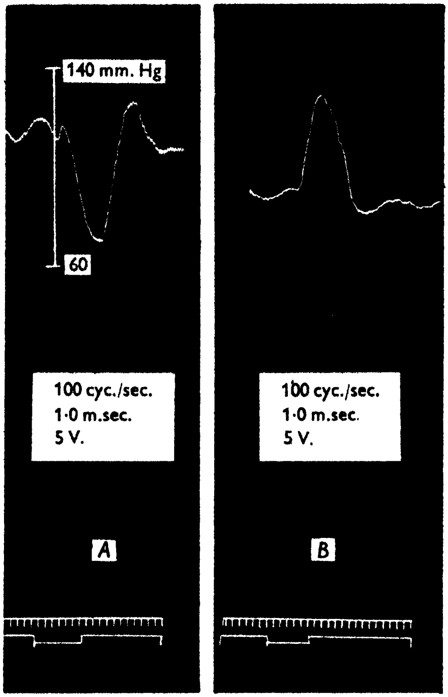

Fig. 7. Decerebrate cat before (A) and 20 min. after intravenous injection of chloralose (0·1 g./kg.). (B) Stimulation of left aortic nerve with 100 cyc./sec., 1·0 msec., 5 V.

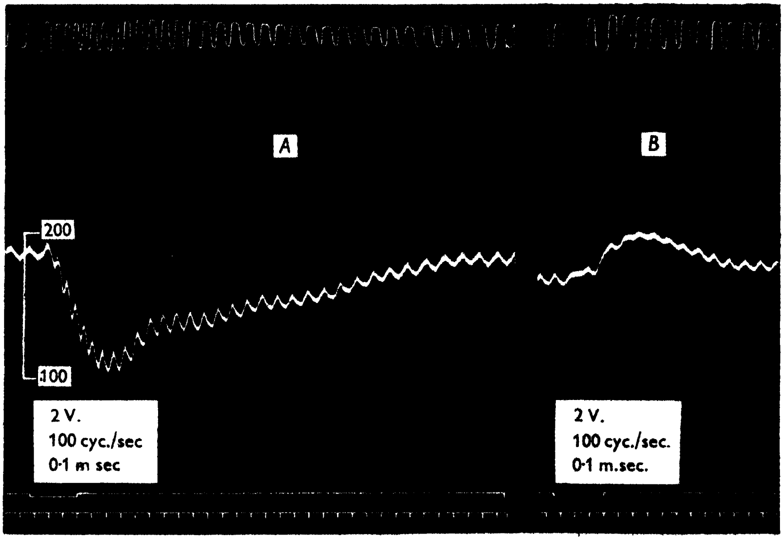

Fig. 8. Cat, nembutal anaesthesia followed by intravenous chloralose. Records from above downwards: respiration, blood pressure, signal, time in 5 sec. Stimulation of left aortic nerve (2 V., 100 cyc./sec., 0·1 msec.) Between *A* and *B*, 0·05 g. chloralose was injected. Record *B* was taken 30 min. after injection.

injections were made into the left ventricle through a cannula inserted via the common carotid artery, the tip of the cannula being pushed through the aortic valve. These experiments were performed in cats with intact and spontaneous respiration. Prior to introduction of the cannula both carotid sinuses were denervated and the aortic nerves were isolated from the vago-sympathetic trunk in the neck.

Section of both vagi, leaving the aortic nerves intact, reduced, but did not abolish, the respiratory response to intraventricular injection of nicotine or lobeline (Neil, Redwood & Schweitzer, 1949*b*) (Fig. 9). In such animals, separate division of the aortic nerves showed that section of the left nerve caused but little further diminution of the response to the drug injection; division of the remaining right aortic nerve, however, completely abolished the hyperpnoeic response to the injection (Fig. 10).

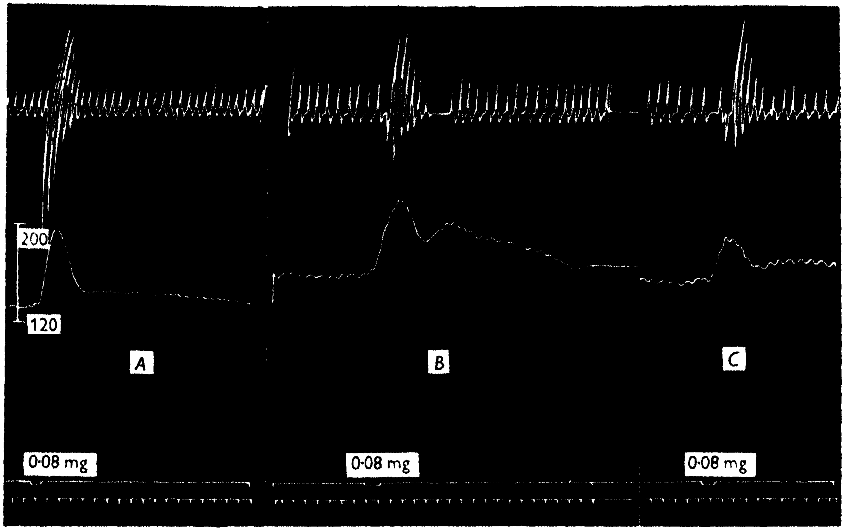

Fig. 9. Cat, chloralose anaesthesia. Records as in preceding figures. At signal 0·08 mg. nicotine was injected intraventricularly. *A*, vagi and aortic nerves intact; *B* vagi cut; *C*, left aortic nerve cut, leaving right aortic nerve intact.

These experiments show that chemoceptor fibres are contained in the trunks of the aortic nerves in the cat. It appears that the right aortic nerve contains a greater number of fibres arising from the aortic body than the left nerve. This difference in the distribution of chemoceptor fibres between the right and left aortic nerves possibly explains the differences in the effects of left and right aortic nerve stimulation in the cat. These experiments have been repeated in the rabbit but, so far, no evidence has been obtained that chemoceptor fibres pass in the aortic nerves. Section of the vagi in the rabbit, after denervation of both carotid sinuses with the aortic nerves remaining intact, abolished the respiratory response to intraventricular injection of lobeline or nicotine.

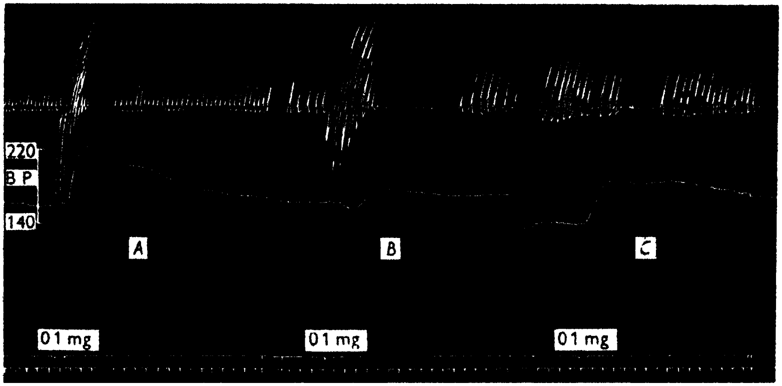

Fig. 10. Cat under chloralose anaesthesia. Records from above downwards: respiration (intratracheal pressure) blood pressure, signal, time in 5 sec. Signal marks the intraventricular injection of 0·1 mg. nicotine. *A*, vagi and aortic nerves intact; *B*, vagi cut; *C*, aortic nerves cut.

DISCUSSION

These results show that the effects on arterial blood pressure of electrical stimulation of the aortic nerves in the cat are dependent upon the type of anaesthesia employed. Under nembutal, given in usual anaesthetic doses, aortic nerve stimulation caused a fall in blood pressure. This response also invariably occurred in decerebrate cats. Subsequent injection of chloralose into cats under nembutal anaesthesia, or decerebrated, usually converted the fall in blood pressure following aortic nerve stimulation into a rise.

Furthermore, in cats under chloralose anaesthesia alone, pressor responses were obtained using stimuli of short pulse duration (1·0 msec., or less). When stimuli of longer pulse duration (10·0 msec.) were employed, the response to stimulation of the left aortic nerve changed to a fall in arterial blood pressure, although stimulation of the right aortic nerve under these experimental conditions produced a rise in pressure.

These differences in the response to stimulation of the left and right aortic nerves of cats are explained by the greater number of chemoceptor fibres running in the right aortic nerve, suggested by the results of intraventricular injection of nicotine or lobeline.

It has been impossible to obtain pressor responses to stimulation of the aortic nerves in the rabbit under experimental conditions which would result in pressor responses in the cat. These negative findings may be related to the absence of chemoceptor fibres in the aortic nerves of the rabbit, as also suggested by the observations of Schmidt (1932) and Gernandt (1946).

The experiments reported in this paper suggest that pressor responses to stimulation of the aortic nerves are due to excitation of chemoceptor fibres. The results of these experiments, and the conclusions drawn from them, are essentially similar to those obtained from experiments on the carotid sinus nerve.

SUMMARY

1. Rectangular wave stimulation of the left or right aortic nerve in rabbits under nembutal or chloralose anaesthesia caused a fall of arterial blood pressure irrespective of the pulse duration of the stimuli employed.

2. Stimulation of either aortic nerve in decerebrate cats or in cats under nembutal anaesthesia caused a fall of arterial blood pressure irrespective of the pulse duration of the stimuli used.

3. Stimulation of the right aortic nerve in cats under chloralose anaesthesia produced a rise in arterial blood pressure irrespective of the pulse duration of the impulses employed.

4. Stimulation of the left aortic nerve in cats under chloralose anaesthesia produced a fall in arterial blood pressure, using pulse durations of 10·0 msec., and a rise in pressure with pulses of 0·1–1·0 msec. duration.

5. Injection of 0·1 g./kg. body weight of chloralose in decerebrate cats or 0·05 or more of chloralose in cats under nembutal anaesthesia converted the depressor response to stimulation of the right aortic nerve into a rise of arterial blood pressure.

6. Evidence is presented by intraventricular injection of nicotine or of lobeline that the aortic nerves of the cat, especially the right, contain chemoceptor fibres from the aortic body. No evidence was obtained that such fibres are present in the aortic nerves in the rabbit.

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THE BLOOD FLOW THROUGH THE HUMAN CALF DURING RHYTHMIC EXERCISE

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It has generally been assumed that the blood flow through human muscle during rhythmic exercise cannot be determined with the plethysmograph (Abramson, 1944). We have, however, been able to make such determinations in special circumstances.

The new method has been used in experiments on the mechanical effect of rhythmic contraction on muscle blood flow. Kramer, Obal & Quensel (1939) showed that maximal rhythmic contractions obstructed the flow in dog's muscle. A. V. Hill (1948*a*, *b*) found that the tension in strongly contracted frog muscle was 100–300 mm. Hg. The work of Grant (1938) and others on sustained human contraction left little doubt that rhythmic contractions *per se* would restrict blood flow in human muscle.

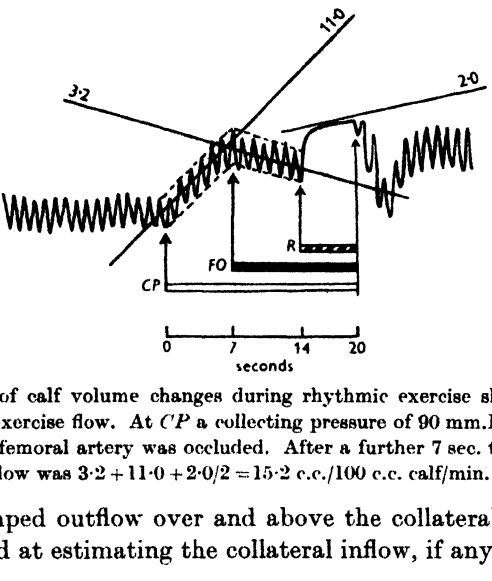
METHODS

Principle for measuring blood flow in the exercising calf

This can best be explained with the aid of Text-fig. 1. The tracing shows a plethysmographic record of the calf volume; the excursions are due to the contractions and relaxations of the calf muscles. The blood flow during rhythmic exercise is obtained as follows:

Text-fig. 1, *CP. A collecting pressure of about* 90 *mm. Hg is applied to the lower part of the thigh by inflation of a pneumatic cuff.* Owing to compression of the veins the venous return is hindered and the calf swells. After the experiment a sloping line is drawn from which the rate of swelling is calculated. In Text-fig. 1 it was 11·0 c.c./100 c.c. calf/min. At first sight this would appear to be the rate of the blood flow during exercise. This is, however, not necessarily the case; the apparent flow may be too small, because, owing to the pumping action of the muscles, some venous blood may escape under the cuff back into the body (Barcroft & Dornhorst, 1949). Measurement of the amount that escapes in this way is the aim of the next procedure.

Text-fig. 1, *FO. Collecting pressure still applied—femoral artery occluded by digital pressure.* The main supply of blood is now cut off and, as Text-fig. 1 shows, shrinkage of the calf takes place. This is usually fairly regular for some seconds at least, and is due to the blood being pumped out under the cuff. After the experiment a sloping line is drawn from which the rate of blood loss is calculated, in Text-fig. 1 it amounted to 3·2 c.c./100 c.c. calf/min. This method of estimating the rate of the pumped outflow may, however, not be quite accurate in all subjects. It will only be accurate if the entry of blood into the calf has been completely stopped by occluding the femoral artery. In some subjects this will indeed be the case, but in others there will still be a small inflow through collateral vessels. The rate of shrinkage of the calf will be a measure of the excess of the pumped outflow over and above the collateral inflow. The final procedure is aimed at estimating the collateral inflow, if any, so that it can be allowed for.



Text-fig. 1. Tracing of calf volume changes during rhythmic exercise showing procedures for determining the exercise flow. At *CP* a collecting pressure of 90 mm.Hg was applied. 7 sec. later, at *FO*, the femoral artery was occluded. After a further 7 sec. the subject relaxed for 6 sec. The mean flow was 3·2 + 11·0 + 2·0/2 = 15·2 c.c./100 c.c. calf/min.

Text-fig. 1, *R. Collecting pressure still applied, femoral artery still occluded—the subject stops the exercise and relaxes the calf muscles completely.* The pumping action of the muscles having ceased the escape of venous blood is now effectively prevented by the collecting cuff. If there is no collateral inflow, the volume of the calf will remain constant, and the recorder will trace a horizontal line. If there is a collateral inflow there will be a gradual increase in calf volume and the line drawn by the recorder will slope upwards.

(i) Let us suppose that during Text-fig. 1, *R*, the recorder had traced a horizontal line, showing that there was no collateral inflow.

In that case the rate of shrinkage of the calf which followed femoral arterial occlusion, Text-fig. 1, *FO* would have been an accurate measure of the rate at which blood was being pumped out under the cuff.

To obtain the blood flow during exercise it would only have been necessary to add the apparent inflow, Text-fig. 1, *CP*, to the pumped outflow, Text-fig. 1, *FO*.

Blood flow during rhythmic exercise = Apparent inflow (Fig. 1, *CP*) + Pumped outflow (Fig. 1, *FO*)
= 11·0 + 3·2
= 14·2 c.c./100 c.c. calf/min.

(ii) In fact, after the subject ceased exercise, the record Text-fig. 1, *R*, slopes slightly upwards. This indicates that there was an inflow of blood from collateral vessels. Its rate was estimated as 2·0 c.c./100 c.c. calf/min.

In this case the rate of shrinkage of the calf which followed femoral arterial occlusion, Text-fig. 1, *FO*, represented the excess of the pumped outflow over the inflow from the collateral vessels. We shall call it the apparent pumped outflow.

To obtain the real pumped outflow the rate of the collateral inflow must be added to the apparent outflow. That is, the rate of the collateral inflow during exercise must be added. This is probably not the same as the rate of the collateral inflow when the subject was completely relaxed, which we know was 2 c.c., Text-fig. 1, *R*. It will be shown below that exercise hinders the blood flow through the calf so the collateral inflow during exercise is likely to be something less than the relaxation collateral inflow—in this case something between 2·0 and 0 c.c. For want of better knowledge we shall assume that exercise halves the rate of the collateral inflow. This is the best estimate that we can make, and a small error will not matter since the collateral inflow is usually small compared with the other values.

We can now calculate the blood flow during rhythmic exercise as follows:

$$\text{Blood flow during rhythmic exercise} = \text{Apparent inflow Fig. 1, } CP + \overbrace{\text{Apparent pumped outflow (Text-fig. 1, } FO) + \text{Collateral inflow during exercise, i.e. } \tfrac{1}{2} \text{ relaxation collateral inflow (Text-fig. 1, } R)}^{\text{Pumped outflow}}$$

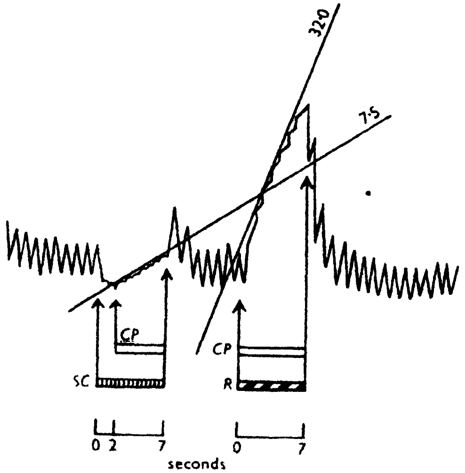
$$= 11{\cdot}0 + 3{\cdot}2 + 2{\cdot}02/2$$
$$= 15{\cdot}2 \text{ c.c./100 c.c. calf/min.}$$

Experimental

The subjects were healthy men aged 20–30. The subject bared his right leg up to the groin and lay on a couch with his head raised. A calf plethysmograph was fitted (cf. Barcroft & Edholm, 1943, 1945). As the absolute level of flow was not important the plethysmograph was air-filled (Barcroft & Edholm, 1946). To take its weight off the leg it was suspended by a rope passing over a pulley to a counterpoise weight. The sock and shoe were put on, as also the ankle cuff for arresting the circulation in the foot, and the collecting cuff above the knee. The subject's position was adjusted; his feet on the pedals of the exercising machine, his shoulders supported by straps, and the plethysmograph clear of the couch.

The pressure point for digital compression of the femoral artery with one finger was then sought; it was at the point of most prominent pulsation about an inch below the groin. While the calf volume was being recorded a test compression was made. It was considered satisfactory if the pulse disappeared, proving that the artery was occluded, and the leg volume decreased. The decrease of leg volume showed that the femoral vein was still patent, as no decrease occurred if the vein was deliberately compressed at the same time as the artery.

Resting flows were taken at 3 min. intervals for 10 min. Exercise then began. A weighted pedal was pressed down once a second to the sound of a metronome. As the pedal moved a pointer travelled over a scale, and as soon as the subject saw it reach a given mark he stopped pressing and relaxed completely. The pressure of the weight on the pedal brought the foot back to the resting position ready for the next contraction. Movement of the pelvis was prevented by making the subject do the same exercise simultaneously with the other foot on another similarly weighted pedal.



Text-fig. 2. Tracing of calf volume changes during exercise showing: Left: procedures for determining the interpolated sustained contraction flow. *SC*, subject kept pedal pressed down; *CP*, collecting pressure. Right: procedures for determining the interpolated relaxation flow. *CP*, collecting pressure; *R*, subject stopped exercise and relaxed completely.

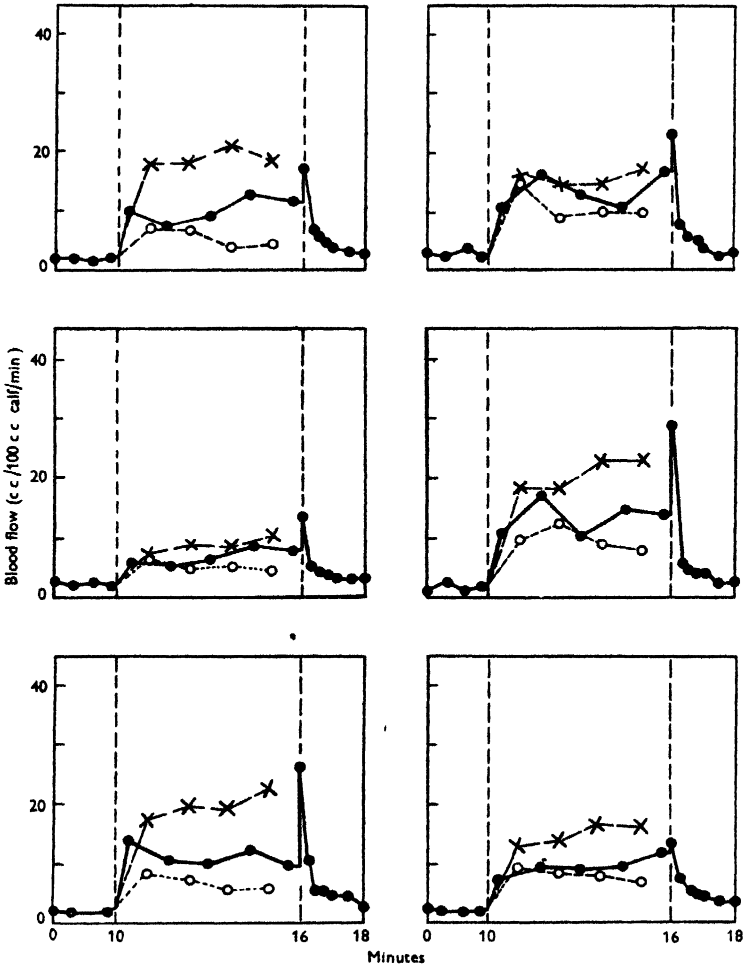
During the exercise, which lasted for 6 min., five records of the exercise flow were taken (Text-fig. 1) and also four records of the flow during brief interpolated periods of relaxation and four during brief interpolated periods of sustained contraction (Text-fig. 2). The ankle cuff was deflated for 5 sec. immediately after each estimation of the exercise flow. This prevented ischaemic pain in the foot while allowing 15 sec. between reinflation of the cuff and the next application of the collecting pressure.

After the subject had rested for ¼ hr. the procedure was repeated with the thrust on the ball of the foot increased from 9 to 15 kg.

RESULTS

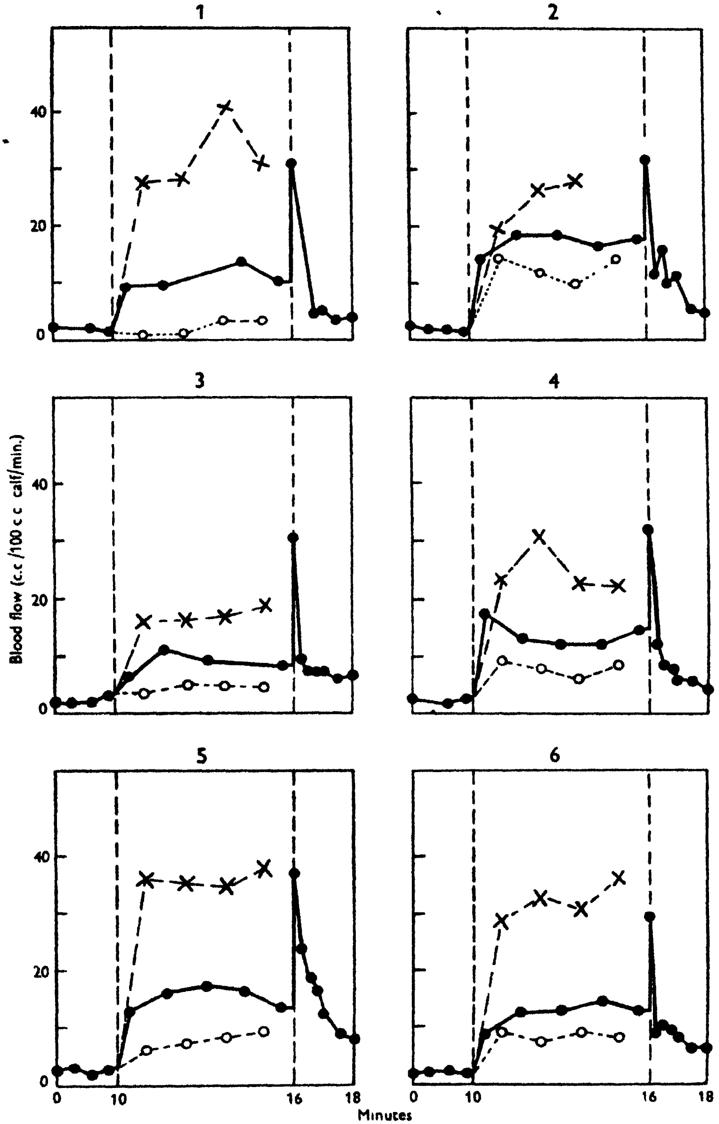
Text-fig. 3 shows the results obtained on six subjects doing the 9 kg. weight exercise. In every experiment the exercise flow was greater than the resting flow. The rate and amount of increase varied a good deal from subject to subject. The exercise flow was greater than the interpolated sustained contraction flow, and less than the interpolated relaxation flow. The immediate post-exercise flow is really analogous to an interpolated relaxation flow, and was greater than the exercise flow. Although the pattern differs the general findings are consistent. Text-fig. 4 shows the results for the exercises with the 15 kg. weight.

The separation of the exercise flows from the interpolated sustained contraction flows and from the interpolated relaxation flows is now very definite. Text-fig. 5 shows tracings from the experiments graphed in Text-fig. 4, nos. 3–5.



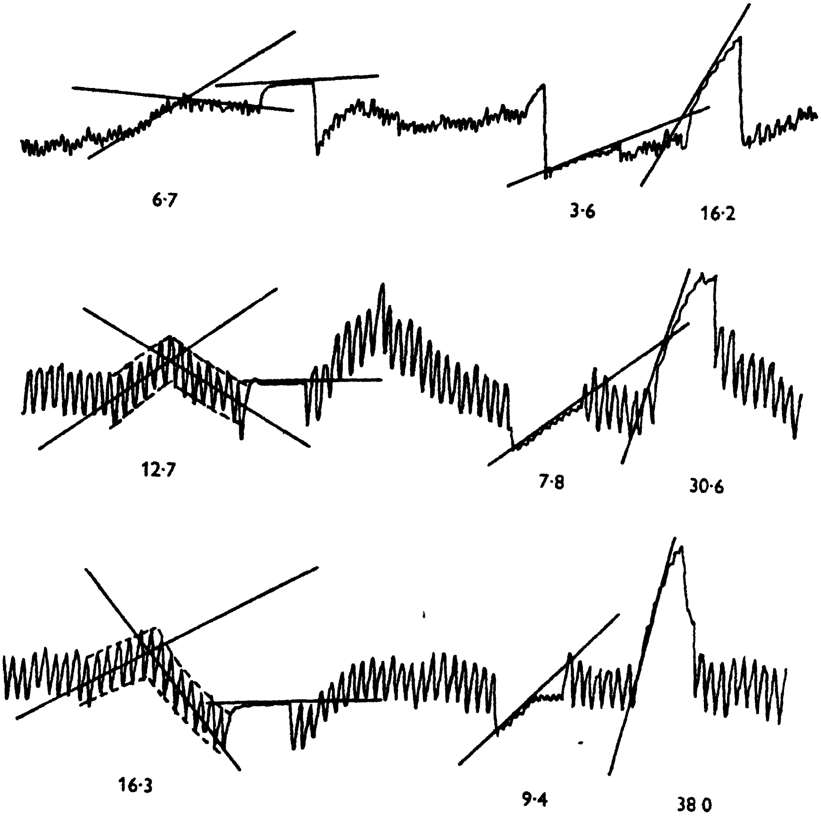
Text-fig. 3. Between the dotted vertical lines: exercise, raising 9 kg. weight once every second. Exercise flow: black dots; interpolated relaxation flows: crosses; interpolated sustained contraction flows: circles.

Text-fig. 6 shows the results of experiments to determine how much the procedures for measuring the exercise flow interfere with the normal reactions of the vessels supplying the active muscle. The top and bottom diagrams are from different subjects. Each diagram shows the blood flow through the calf recorded for 2 min. after each of four spells of exercise with 15 kg. weight,



Text-fig. 4. Between the dotted vertical lines: exercise raising 15 kg. weight once every second. Exercise flow: black dots; interpolated relaxation flows: crosses; interpolated sustained contraction flows: circles.

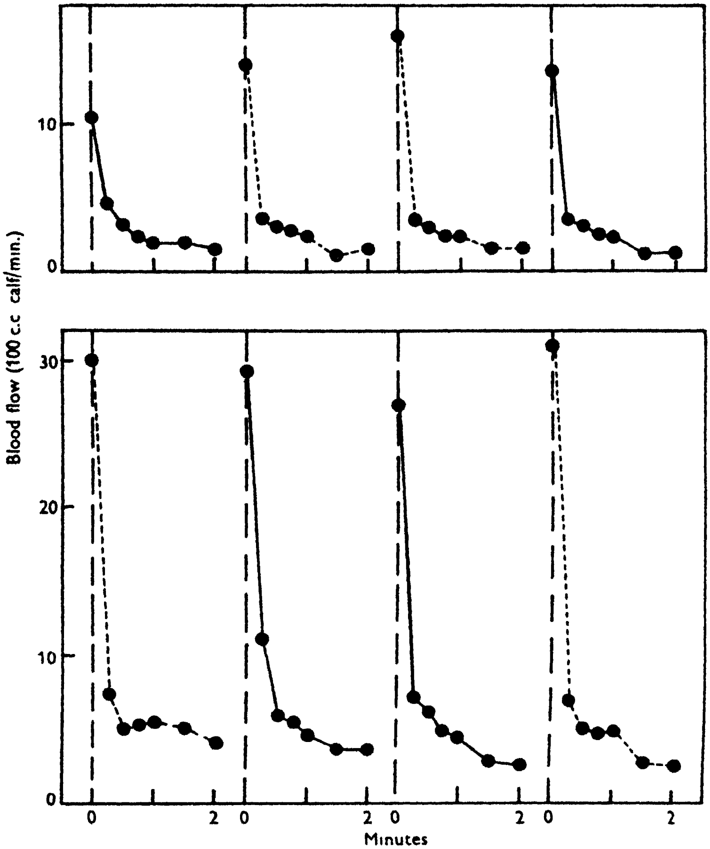
performed, with suitable intervals of rest, in the order in which they are charted. Each continuous line curve was recorded after 2½ min. continuous exercise, each broken line curve after a similar exercise during which three estimates of the exercise flow were made. It will be seen that the immediate post-exercise flow was somewhat greater when the exercise had been punctuated by these procedures, but that subsequent post-exercise flows are not increased.



Text-fig. 5. Tracings taken during exercise in the experiments shown in Text-fig. 4, nos. 3–5 showing typical patterns. Each tracing shows a record of the procedures for estimating a blood flow during rhythmic exercise, during interpolated sustained contraction and during interpolated relaxation. The figures under each record are calculated as explained in the text.

DISCUSSION

Grant (1938), Dolgin & Lehmann (1930), Barcroft & Millen (1939) and others have shown that strong sustained contraction of human muscle mechanically decreases the blood flow. It seemed that strong rhythmic contraction must do so too. The experiments just described confirm this. They show conclusively that immediately the contraction stopped the blood stream, unhindered, quickened.



Text-fig. 6. Each curve represents the rate of blood flow through the calf during 2 min. immediately following a $2\frac{1}{2}$ min. period of exercise. For further discussion see text.

Table 1 shows some approximate figures for the blood flow in the gastrocnemius soleus before, during, and after exercise.

TABLE 1. Average blood flow through the gastrocnemius soleus muscle

Weight on pedal (kg.)	9	15
Initial resting flow (c.c./100 c.c. muscle/min.)	2·5	2·5
Flow towards end of exercise (c.c./100 c.c. muscle/min.)	28	30
Flow immediately after exercise (c.c./100 c.c. muscle/min.)	45	77

The figures were calculated from the averaged results of the calf flows shown in Text-figs. 3 and 4. The volume of the gastrocnemius soleus was taken as

740 c.c. (Reys, 1915); about three-quarters of it, say 550 c.c., was judged to have been in the plethysmograph. The total volume of calf enclosed averaged 1400 c.c., of which the gastrocnemius soleus, therefore, accounted for about $\frac{550}{1400} \times 100 = 40\%$. The resting calf flow averaged 2 c.c., about half of which must have been going through the gastrocnemius, and the rest, namely 1 c.c., through the skin, bone and other muscles. The flow through the other tissues was taken as remaining substantially unaltered by the exercise. Accordingly the gastrocnemius muscle flows in the table were obtained by subtracting 1 c.c. from the averaged calf flows and multiplying the difference by $\frac{100}{40}$, i.e. by $2\frac{1}{2}$.

Table 1 emphasizes the following points:

(i) In the 9 kg. exercise the mean flow was 62% of the post-exercise flow. In the 15 kg. exercise it was 39%. The mechanical restriction of the flow with increasing load is most striking.

(ii) The mean flow in the two exercises was about the same. This is probably because mechanical hindrance was less in the 9 kg. exercise and although vasodilatation was less too, relatively more blood got through.

Finally, the following consideration is of some interest. Suppose that all the muscles in the body were working hard and had the same mean flow, 30 c.c., as that in the 15 kg. exercise. Total muscle blood flow would be about $8\frac{1}{2}$ l./min. (body wt. 70 kg.; 40% muscle, Bardeleben, 1912). Cardiac output would have been about 13 l. This is very low compared with the 20 l. which Hill (1948*a*, *b*) calculated to be the minimum for running at about 10 m.p.h. Difference in body temperature and arterial blood pressure may be factors in the discrepancy, and probably more important still, difference in the kind of movement. In running, relaxation may have lasted relatively longer than in pedalling. Further experiments would be interesting.

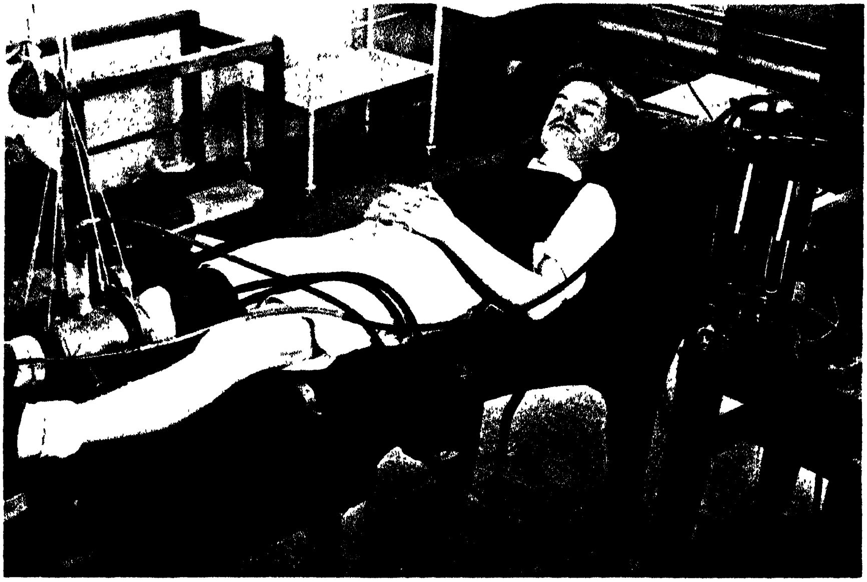
SUMMARY

1. A plethysmographic method is described for determining, approximately, in special circumstances, the blood flow through the human calf during rhythmic contraction of the gastrocnemius soleus.

2. Previous work showing that rhythmic contractions *per se* must engender a mechanical resistance to the passage of blood through human muscle is confirmed.

3. During an exercise in which the subject pressed a weighted pedal once a second, the mechanical hindrance of the contractions reduced the flow to 40% of what it would otherwise have been.

The authors thank Prof. A. D. M. Greenfield for a suggestion that led to this investigation, Drs H. E. de Wardener and H. M. McClatchey for assistance, and Mr J. Dalrymple for technical assistance. They are also very much indebted to the students at St Thomas's Hospital Medical School who acted as subjects. The expenses were partly defrayed by the Medical Research Council.



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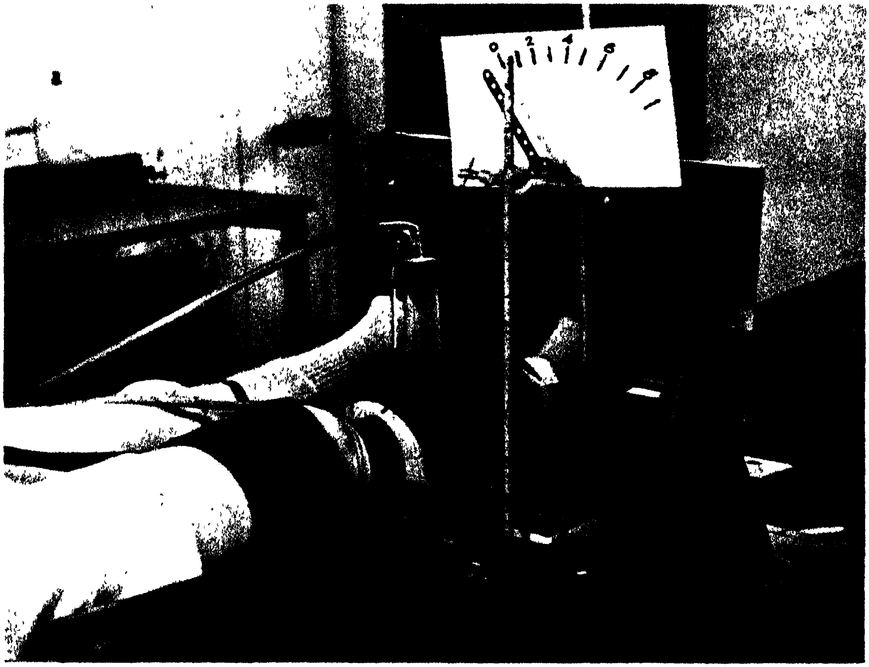

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EXPLANATION OF PLATE

The plates show the plethysmograph; sling and counterpoise weight (A); collecting cuff on the lower part of the thigh; ankle cuff for arresting the circulation in the foot; heels resting on fixed supports; feet on pedals; horizontal bars at right angles to pedals: each is pivoted separately where pedal and bar join; weights on bars for adjusting work done; pointer actuated by movement of pedal and bar on right side (B); stops at head end of bar, one for taking weight off pedal during relaxation, the other to prevent movement of pointer beyond end of scale (A); metronome (B); straps supporting subject's shoulders (A); control board on end of kymograph table (A). The subject is looking at the pointer.

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THE ACTION OF D-TUBOCURARINE CHLORIDE ON FOETAL NEUROMUSCULAR TRANSMISSION AND THE PLACENTAL TRANSFER OF THIS DRUG IN THE RABBIT

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The employment of curare to procure muscular relaxation during anaesthesia is no longer a novelty, and there are many clinical reports of its widespread use in surgery and the treatment of some spastic conditions. However, before curare may be used, as an adjunct to anaesthesia during Caesarian section, definite information is desirable concerning the action of curare on foetal neuromuscular transmission and the permeability of the placental barrier to curare.

Preyer (1885) believed that the foetal neuromuscular junction did not show adult characteristics until the end of the gestation period. His crude curarine paralysed newborn guinea-pigs and post-mature foetuses, but doses adequate to curarize an adult guinea-pig failed to paralyse full-term or more immature foetuses. Other workers have assumed that curare blocks transmission at the foetal neuromuscular junction in a manner exactly comparable to the effect of the drug on voluntary muscle in the adult animal. Coghill (1934) studying the development of somatic movements in the killi fish (*Fundulus heteroclitus*), Gonzalez (1935) investigating the innervation of skeletal muscle in rat foetuses, and Kuo (1939) in a study of the development of the nervous system in chick embryos, all used crude curarine preparations to distinguish between the myogenic and neurogenic origin of motor activity. The paralyses observed might have been due to handling of the material and the subsequent development of anoxia, for in mammalian experiments the integrity of the placental circulation is readily impaired by handling of the uterus.

Preyer was the first to record observations upon the placental transfer of curare. In guinea-pigs the crude drug passed very readily from the foetal to the maternal circulation, but as his embryos were not susceptible to curare he possessed no means of testing whether it would cross the placental barrier in the reverse direction. However, following the introduction of curare into anaesthesia by Griffiths & Johnson (1942), its use in Caesarian section revives this question of the transfer of the drug from the maternal to the foetal circulation, together with the problem of the susceptibility of the human foetus to curare. In two recent clinical accounts, standard preparations of curare have been used. Whitacre & Fisher (1945) published observations on one hundred Caesarian sections, anaesthetized with cyclopropane in which muscular relaxation was obtained with Squibb's Intocostrin. As a 'slight depression' of the infant was encountered more frequently than in babies from Caesarian sections where a spinal or local anaesthetic alone was used, they concluded that the use of curare in these cases needed further investigation to assess its safety. Gray (1947) recorded a further thirty cases in which he induced anaesthesia in the mother with Kemithal and maintained it with cyclopropane. Muscular relaxation was produced by 15 mg. D-tubocurarine chloride (Burroughs Wellcome 'Tubarine'). He found that the infants cried lustily as soon as the head was delivered and therefore considered the full-term foetus insusceptible to curare.

Existing evidence concerning the action of curare on foetal neuromuscular transmission is therefore limited and discordant; these discrepancies may be ascribed to the use of crude curare preparations of varying composition and potency and to the existence of foetal anoxia. The present observations were made to determine the action of a standardized preparation, D-tubocurarine chloride, on foetal neuromuscular transmission and to investigate the passage of this drug across the placental barrier from the maternal to the foetal circulation and in the reverse direction.

METHODS

Action of D-tubocurarine chloride on foetal neuromuscular transmission

This was investigated by two methods; first, by intravascular injection of lively foetuses having a good placental circulation; and secondly, by observations on isolated phrenic-nerve-diaphragm preparations. The experimental material consisted of 28–31-day rabbit foetuses, 50–60-day guinea-pig foetuses and 20–22-week human foetuses.

Intravascular injection. Pregnant rabbits were anaesthetized with urethane given intravenously, 2 g./kg. body wt.; the arterial blood pressure was recorded with a mercury manometer throughout the observations, since it had been noticed previously that if the blood pressure fell below 80–70 mm. Hg the foetuses soon became moribund. The pregnant guinea-pigs were also anaesthetized with urethane given intraperitoneally (2 g./kg. body wt.); the arterial blood pressure was not recorded. A foetus was located and carefully exposed through a longitudinal incision made in the uterine wall from crown to rump of the foetus; in this way there is very little disturbance of the rest of the uterus and little bleeding, as the uterine wall contracts down around the incision. The placenta was examined quickly and judged to have an adequate circulation on both the maternal and the foetal side if the colour was a characteristic purple red and the umbilical vein noticeably more red than the umbilical arteries. During the examination of the foetus it was held so that its attachment with the placenta was in no way strained. A few of the experiments were performed in a saline bath and these results were in complete agreement with the others. A total of twelve rabbit foetuses and six guinea-pig foetuses received doses varying from 10 to 100 μg. D-tubocurarine chloride, in 0·1 ml. saline, injected into the umbilical vein. When the foetuses became quiescent the phrenic and sciatic nerves were exposed and pinched with fine forceps. This test for neuromuscular transmission was chosen to obviate any spread of stimulus to the muscle. Finally, the corresponding muscles were stimulated directly with weak induction shocks.

Isolated phrenic nerve diaphragm preparations. These preparations from rabbit and guinea-pig foetuses are about two-thirds the size of the average rat diaphragm preparation, the muscle is thinner and the nerve very delicate. Observations were made at 37° C. with the muscle immersed in Tyrode containing 0·2 g. % glucose, adjusted to pH 7·4–7·6 with N/10 HCl, and aerated with oxygen containing 5 % CO_2. The adjustment of pH, together with aeration by oxygen and carbon dioxide, was essential to the success of the preparation, which under these conditions was extremely robust, lasting 4–6 hr. The method of Holmes, Jenden & Taylor (1947) was used to suspend the muscle so that it could be stimulated directly while immersed. A 2 mfd. condenser charged to 36–120 V. was used for stimulation. The nerve lay over electrodes held just above the surface of the Tyrode and was stimulated from 0·01 mfd. condenser charged to 12 V. The muscle and nerve were stimulated alternately every 10 sec. D-Tubocurarine chloride was added in concentrations varying from 1 to 10 μg. per ml. of bath and the time noted for the onset of neuromuscular block and the completion of the block. These times were compared with those for the action of the same concentration of the drug upon the adult rat phrenic nerve diaphragm preparation. The activities of prostigmine and potassium ions as antagonists to the action of curare were also investigated.

Four 20–22-week human foetuses were obtained from termination of pregnancies. Phrenic-nerve-diaphragm preparations are readily made, and the muscle is found to be about four times as thick as the adult rat preparation, and the nerve about double in diameter. The preparations were set up in the bath at intervals ranging from 1½ to 3½ hr. after removal of the foetus from the uterus. Initially, in each case, the muscle responded poorly to direct stimulation and not at all to stimulation of the phrenic nerve. A full response was not obtained by either method of stimulation until the preparation had been in Tyrode for 1 hr., thereafter it responded well for 17–20 hr.

Placental transfer of curare

The passage of D-tubocurarine from the maternal to the foetal blood and from the foetal to the maternal blood stream was investigated in does 28–31 days pregnant. The general experimental procedure was similar to that described above with the following differences:

Transfer from maternal to foetal circulation. Before the initial injection of curare into the mother, one foetus was examined as a control and the simple pinch test for neuromuscular transmission performed. This foetus was then discarded and the placenta gently pulled away from the uterine wall which contracted down in this area with very little bleeding. The doe received an initial dose of 0·5–0·75 mg. D-tubocurarine intravenously, which was just sufficient to inhibit respiratory movements completely in the average 3 kg. rabbit for ½ hr. Subsequently, 0·5 mg. D-tubocurarine was given intravenously at half-hourly intervals after the first injection. The success of the experiment depended upon keeping the maternal blood fully oxygenated: therefore oxygen in a closed circuit was used in maintaining artificial respiration. Foetuses were examined at half-hourly or hourly intervals, throughout the average 3 hr. experimental period, after the first injection of curare.

Transfer from the foetal to the maternal circulation. The maternal respiratory movements were recorded from the diaphragm slip; contractions of the gastrocnemius muscle, elicited by stimulation of the peripheral end of the sciatic nerve, were recorded at 10 sec. intervals. (The discharge from a 2 mfd. condenser charged to 36–120 V. was used for stimulation.) A foetus was exposed and 0·5–4 mg. D-tubocurarine in 0·1 ml. was injected into one umbilical artery towards the placenta. As the needle was withdrawn the artery was ligatured above and below the puncture to prevent leak of either blood or curare. The foetus was carefully replaced and the abdominal wound closed. After about half an hour the placenta was inspected to make sure that it was still adherent to the uterine wall and that there was no leak of either maternal or foetal blood.

RESULTS

Action of D-*tubocurarine chloride on foetal neuromuscular transmission*

Intravascular injection. 10–100 µg. D-tubocurarine chloride intravenously caused complete paralysis in 2–3 min. both in rabbit and guinea-pig foetuses. This is slower than the onset of paralysis in the adult animal, and is possibly due to slowing in the cardiac rhythm and a relatively slower circulation in the foetus caused by haemorrhage from the umbilical vein which occurred, however sucesssful the injection. D-Tubocurarine (10 µg.) injected intravenously in a 50 g. foetus is weight for weight equivalent to the 0·5–0·75 mg. D-tubocurarine chloride which is just sufficient to inhibit respiration in the average 3 kg. rabbit.

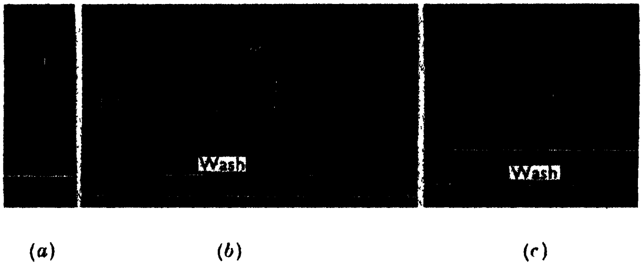


Fig. 1. Phrenic nerve and diaphragmatic muscle stimulated alternately every 10 sec. with condenser discharges. (*a*) 57-day guinea-pig foetus; D-tubocurarine 2 µg./ml. in bath. Complete paralysis in 3½ min. (*b*) 31-day rabbit foetus; D-tubocurarine 2 µg./ml. in bath. Complete paralysis in 2½ min. (*c*) 28-day rabbit foetus; D-tubocurarine 1 µg./ml. in bath. Complete paralysis in 5 min. In all figures 10 sec. time is marker in every experiment.

Ten minutes after delivery by Caesarian section, one 22-week human foetus, weighing 550 g., received an injection of 2 mg. D-tubocurarine chloride into the umbilical vein, which was followed by 1 mg. into the jugular vein 5 min. later. Initially, the cord was pulsating 92/min. but otherwise the foetus was not very lively, responding only by sluggish twitches to tapping of the skin. Pinching the freed sciatic produced a sustained contraction from the gastrocnemius which was abolished 2 min. after the intrajugular injection of D-tubocurarine. The pulse rate at this time had fallen to 76/min. This foetus received weight for weight about thirty times the threshold dose required to inhibit respiration in rabbit and guinea-pig foetuses.

Isolated diaphragm phrenic nerve preparations. Typical results for rabbit and guinea-pig foetuses are shown in Fig. 1. The height of the maximal response to direct stimulation of the muscle was always slightly greater than the maximal response to indirect stimulation. The average time for complete neuromuscular block was 5 min. with a concentration of 1 µg. D-tubocurarine/ml. in the bath and 2½ min. with a concentration of 2 µg./ml. These times are reproducible and identical with those for the adult rat diaphragm-phrenic-nerve preparation.

In all preparations, however, complete paralysis is always slower in onset following the first dose of D-tubocurarine.

In all four human preparations paralysis was slow in onset and in reaching completion (Fig. 2); complete recovery when the bath was changed took 1–1½ hr. To support the view that these results were due to delay in penetration through the thicker connective tissue and muscle, preparations of approximately the same size were made from 1 kg. rabbits; even when freshly prepared these diaphragms did not give any regular response to either direct or indirect stimulation, and after 1 hr. they ceased to respond.

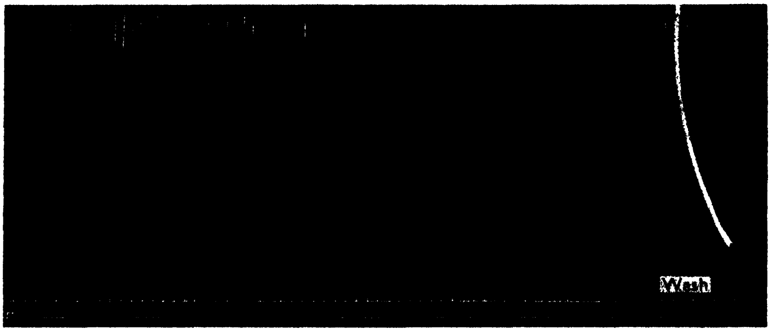
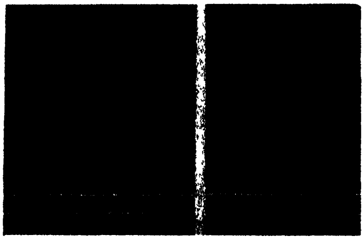


Fig. 2. Phrenic nerve and diaphragmatic muscle stimulated alternately every 10 sec. with condenser discharges. 22-week human foetus; D-tubocurarine 5 μg./ml. in bath; complete inhibition in 10 min.



(a) (b)

Fig. 3. Phrenic nerve and diaphragmatic muscle stimulated alternately every 10 sec. with condenser discharges. 55-day guinea-pig foetus. Reversal of paralysis due to 20 μg. D-tubocurarine in 40 ml. bath, (a) 5 μg. prostigmine, (b) 28 mg. potassium chloride.

Each record shows that D-tubocurarine chloride apparently causes a small decrease in the response of the muscle to direct stimulation, but this is of doubtful significance since direct stimulation of the muscle immersed in Tyrode is not perfectly satisfactory; when the diaphragm is stimulated the stimulus must also excite the phrenic nerve as it enters the muscle and there is frequently summation of these two stimuli. The optimal stimulus for the muscle had always to be found in the presence of curare. The neuromuscular block caused by D-tubocurarine was reversed by prostigmine and potassium ions in diaphragms from rabbit, guinea-pig and human foetuses. Fig. 3 shows the reversal of paralysis, due to 20 μg. D-tubocurarine in a guinea-pig preparation, by 5 μg. prostigmine and 28 mg. potassium chloride. Here again there is complete agreement with the results in the mature animal.

Placental transfer of curare

Transfer from maternal to foetal circulation. Transfer of curare from mother to foetus was never demonstrated; foetuses were very lively when exposed, provided the maternal blood pressure did not fall below 80 mm. Hg, but they became quiescent or moribund if the maternal blood pressure fell below 70 mm. Hg for so short an interval as 10 min.

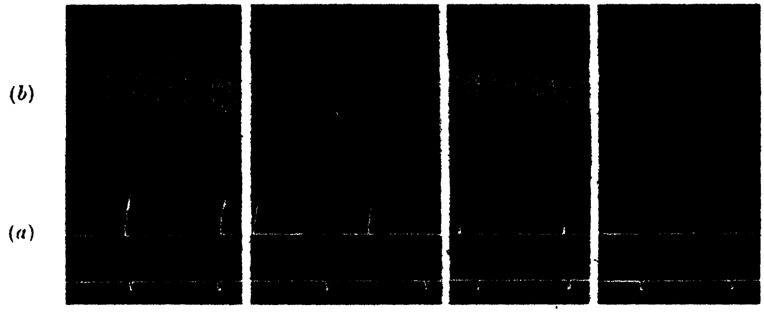


Fig. 4. Effect of 1 mg. D-tubocurarine injected into umbilical artery in the rabbit. (*a*) Response of maternal gastrocnemius muscle to stimulation of sciatic nerve, inhibited in 11 min.; (*b*) movements of diaphragm slip. Maternal blood pressure 110–120 mm. Hg. Intervals represent 5, 2 and 2 min.

Transfer from the foetal to the maternal circulation. The passage of D-tubocurarine from the foetal blood to the maternal blood stream was shown in seven pregnant rabbits and a typical result is shown in Fig. 4, where 0·5 mg. D-tubocurarine injected into the umbilical artery of one of the foetuses was sufficient to inhibit the response of the maternal gastrocnemius muscle to stimulation of the sciatic nerve; 1·0 mg. D-tubocurarine was necessary to decrease her respiratory movements. Inhibition of these responses was complete in times varying from 5 to 30 min. after the foetal injection. Transfer of the drug took place equally well whether the maternal blood pressure was 40 or 100 mm. Hg, but the excitability of the uterine muscle was very important since transfer took longer in two rabbits, 31 days' pregnant, in which very active contraction of the uterus accompanied any handling, and in one experiment no demonstratable quantity of D-tubocurarine crossed into the maternal blood stream during 1 hr.

DISCUSSION

In the present series of experiments there was no demonstrable difference between the sensitivity of the adult and that of the late-term rabbit or guinea-pig foetus to D-tubocurarine injected intravenously. Moreover, the sensitivity of both these isolated foetal rabbit and guinea-pig diaphragm-phrenic-nerve preparations is very similar to those of the adult rat to D-tubocurarine, which confirms Wien's (1948) results that so far as this substance is concerned there is little species difference. Indeed, since these late term foetuses are capable of complicated and co-ordinated reflex movements, the pathways for which are laid down early in development, it would be expected that the motor end-plate should possess properties similar to those of the adult. The increased excitability of the muscle produced by both prostigmine and potassium and the antagonism of prostigmine and potassium ions to the paralysis caused by curare in these foetal muscle-nerve preparations, further bears this out.

Although the 20–22-week human foetuses studied were, developmentally, very much younger than the animal material, similar blocking of the myoneural junction by curare was observed, together with the reversal of this paralysis by prostigmine and potasssium ions. The human diaphragm-phrenic-nerve preparation had a lower sensitivity to the drug than any of the others studied; histological preparations of this human muscle show that it is about four times as thick as the foetal rabbit and adult rat diaphragms examined and surrounded by a very much thicker connective tissue sheath, both of which would delay penetration of the drug. The one observation on a 22-week human foetus also showed that neuromuscular transmission was abolished by intravenous D-tubocurarine, though the total dose, 2 mg. into the umbilical vein plus 1 mg. into the jugular vein, was thirty times that required to paralyse rabbit and guinea-pig foetuses. In contrast, Bourne (1947) reported that 2·5 mg. D-tubocurarine injected into the umbilical vein in one 19-week human foetus produced tetanic spasm of the flexor muscles of the limbs which was followed by death of the foetus a few minutes later. Convulsions have been observed in the cat after intracisternal and intrathecal injection of D-tubocurarine (von Euler & Wahlund, 1941).

The absence of transfer of D-tubocurarine from the maternal to the foetal blood stream in rabbits in sufficient concentration to cause any paralysis of the foetal muscles is in accordance with the clinical experience of Gray (1947) who used D-tubocurarine for abdominal relaxation during Caesarian section, and the observation made by Harroun, Beckert & Fisher (1947) who performed a Caesarian section in a bitch, of unspecified weight, that had received 60 mg. curare. (0·5–0·75 mg. D-tubocurarine given intravenously in a 3 kg. rabbit is equivalent weight for weight to the 15 mg. injected intravenously in the average 60 kg. woman prior to Caesarian section, and represents a concentration of

3 μg./ml. circulating blood; this dose is sufficient to inhibit respiratory movements in the urethanized rabbit rapidly and completely, but in the anaesthetized human there is only slight depression of respiration accompanying the immediate muscular relaxation.) The duration of the average Caesarian section up to the time when the child is delivered is 20–30 min., and the placental circulation must be good until the uterus is incised. In our experiments, it might be objected that the placental circulation was poor and that no curare reached the foetal villi. However, this view may be discounted on several grounds. First, the placentae were a good purple red in colour with the umbilical vein markedly more red than the umbilical arteries; secondly, the foetuses, when exposed, were lively throughout the experimental period. Finally, in some experiments radioactive phosphorus was given intravenously, as the disodium hydrogen salt, with the first injection of D-tubocurarine to the mother and followed by a further dose after 1 hr. (a total of 25 μc. P_{32} equivalent to not more than 0·8 mg. total phosphorus). Radioactive phosphate was transferred across the placental barrier in every case and the foetal blood levels during the experimental period averaged 88% of the maternal concentration. This evidence indicates that the placental circulation was intact during the experimental period and it thus appears that a circulating drug would have ample opportunity to come into contact with the foetal placenta. The absence of curarization of the foetuses, does show that this structure is an effective barrier against the passage of D-tubocurarine from the maternal to the foetal blood stream. No estimation of the circulating D-tubocurarine, nor that present in other body fluids in the mother or foetus has yet been attempted. The rapid disappearance of the drug from the maternal circulation, the presence of an inhibitor in the blood stream or at the placental barrier might all account for the absence of its appearance in the foetal blood stream. Again, there may be a very powerful inhibitor present in the foetal blood stream, or the drug may pass the barrier so slowly that it is excreted by the foetal kidney before an effective circulating concentration can accumulate.

The ready passage of D-tubocurarine from the foetal to the maternal blood stream amply confirms Preyer's results in guinea-pigs towards the end of the gestation period, but our observations differ from his in one respect. Very high concentrations of curare must of necessity be present in the foetal blood for sufficient to cross the barrier to cause any demonstratable block on the maternal side; in our experiments these large doses of curare injected into the umbilical artery always caused instantaneous and complete paralysis of the rabbit or guinea-pig foetus together with a bradycardia which was followed by complete cessation of the heart beat within 20–30 min. Preyer, on the other hand, found that his foetuses remained very active after large doses of crude curare. This passage of D-tubocurarine chloride from the foetal to the maternal blood stream has only been observed with a calculated concentration of 140 μg./ml. circulating foetal blood (assuming that the average 50 g. foetus has a blood volume of approximately 3·5 ml.). This concentration is forty-seven times that calculated to be present in the maternal circulation with a curarizing dose, i.e. 3 μg./ml. It would be interesting to perfuse the placenta artificially on the foetal side with blood containing 3 μg./ml. D-tubocurarine in sufficient volume so that 0·5–0·75 mg. D-tubocurarine might cross the placental barrier to produce paralysis in the mother.

SUMMARY

1. The neuromuscular mechanism of 28–31-day foetal rabbits and 50–60-day foetal guinea-pigs is blocked by D-tubocurarine chloride; isolated diaphragm-phrenic-nerve preparations from these embryos are as sensitive to the drug as a similar isolated preparation from the adult rat.

2. The neuromuscular mechanism in the 20–22-week human foetus is also blocked by D-tobucurarine chloride; the isolated diaphragm phrenic nerve preparation is, however, less sensitive to the drug than the adult rat diaphragm.

3. The neuromuscular block caused by D-tubocurarine chloride is reversed by prostigmine and potassium ions in rabbit, guinea-pig and human foetal preparations.

4. D-Tubocurarine chloride given intravenously over a maximum 3 hr. period to does 28–31 days' pregnant, in a dose equivalent weight for weight to the therapeutic dose in man (3 μg./ml. blood), has not been shown to cross the placental barrier in sufficient quantity to cause respiratory arrest in the foetus.

5. D-Tubocurarine chloride crosses the placental barrier from the foetus to the mother when present in the foetal blood stream in an approximate concentration of 140 μg./ml.

We wish to thank Prof. H. Barcroft for facilities, criticism and encouragement; Dr Ian Donald, of St Thomas's Hospital, and Dr Doreen Daley, of St Helier's Hospital, for making available the human foetuses; and Dr G. Popják, who kindly initiated us into the use of radioactive tracers. We are indebted to the Medical Research Council for the generous supplies of radioactive phosphorus.

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THE EFFECTS OF COOLING AND WARMING ON THE VITAL CAPACITY, FOREARM AND HAND VOLUME, AND SKIN TEMPERATURE OF MAN

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The changes in peripheral blood flow which follow cooling and warming in animals and man have been frequently studied, and there is no doubt that cooling usually reduces the peripheral blood flow while warming increases it, although there are a few, almost paradoxical, exceptions (Lewis, 1930; Pickering, 1932). The question where the blood is to be found when it is not in the peripheral vessels has, however, been consistently ignored or left unanswered, even by those who recognized that alternative pathways must exist (Maddock & Coller, 1933; Machle & Hatch, 1947). This is all the more striking since those working on long-term changes of the circulating blood volume following acclimatization to changes of temperature have considered the problem of blood storage (Barcroft, 1934; Bazett, Sunderman, Doupe & Scott, 1940), and since it has been clearly shown that the changes of peripheral blood flow which follow on cooling and warming must be very great (Barcroft & Nagahashi, 1921; Barcroft & Edholm, 1943). Quite apart from compensatory adaptations of the heart rate (Davy, 1845; Haldane, 1905), the cardiac output (Barcroft & Marshall, 1923), and the circulation rate (Smith, Allen & Craig, 1940), it might be expected that some redistribution of blood would occur when animals and men are exposed to sudden changes of temperature. Rein (1931) noticed that cooling greatly increased the mesenteric blood flow of dogs, but he did not consider this to be a compensation for a reduced peripheral blood flow. Müller and his pupils (Müller, 1905; Müller & Siebeck, 1907; Müller & Veiel, 1910; Glamser, 1911) studied this problem systematically, but their work has apparently been ignored. In a series of thorough experiments, and by a variety of methods applied to men and animals, these workers showed that the reduction of the peripheral blood flow which followed cooling was accompanied by an increase of the intra-abdominal and the cerebral blood flow, and that the opposite happened as a result of warming. Müller (1905) was unable to demonstrate that the human lungs played any part in blood redistribution, but Sjöstrand (1935) found that the lungs of mice which had been killed after cooling weighed more than the lungs of those which had been killed after warming and he suggested that the lungs could 'retain blood for the purpose of regulating heat'.

The blood content of the lungs has been studied by many workers. Daly (1928) showed that the quantity of blood in the lungs was variable. Evidence was subsequently produced which suggested that the lungs could act as a reservoir for blood both in animals (Hochrein & Keller, 1932) and men (Hamilton & Morgan, 1932), and the latter findings were repeated in a statistically significant series by Dow (1939). Experience with patients suffering from cardiac insufficiency (Peabody & Wentworth, 1917; Binger, 1923) and investigations by Budelmann (1937, 1938); Asmussen, Christensen & Sjöstrand (1939); McMichael & McGibbon (1939) and Jonsell & Sjöstrand (1941) confirmed the view that the volume of the air in the lungs varied inversely with that of the blood. Finally, Glaser & McMichael (1941) showed, in a statistically significant series, that removal of 380 ml. of blood from the human circulation was followed by an average increase of 153 ml. in the vital capacity and 181 ml. in the total air capacity of the lungs, which suggested very strongly that comparable changes had happened in the volume of the pulmonary blood. This seemed reasonable since it had been estimated that the human lungs held about 1 l. of blood (Blumgart & Weiss, 1929) or a little more (Plesch, 1937), and since Daly, Foggie & Hebb (1940) had observed changes of about 20 % in the total blood volume of the lungs of dogs. Changes of the alveolar CO_2 concentration have also been claimed to run parallel with changes of the blood content of the lungs (Mackay, 1943), but Roughton (1945) estimated that the average amount of blood contained in patent human lung capillaries and taking part in respiratory exchanges was only about 65 ml. at rest and 95 ml. during heavy work. These calculations appeared to refute all the above observations and estimations, for the lungs could not store more blood than they held. On the other hand, there is some evidence that, at any rate in animals, only part of the blood in the lungs is taking part in the respiratory exchanges at any one time. Sjöstrand (1935) has shown that mice and guinea-pigs have wide vascular passages in their lungs, and Prinzmetal, Ornitz, Simkin & Bergman (1948) have demonstrated in larger laboratory animals that these passages are 20–40 times wider than the capillaries. If such vascular spaces did exist in man it would be possible to reconcile most of the above findings with each other. Thus, in the absence of clear evidence to the contrary and in view of all the circumstantial evidence quoted above, it seems justifiable to conclude that the lungs may exert a reservoir function (using the word to mean any vascular region which can accommodate more or less blood to suit the needs of other parts of the body), and that changes in the volume of air in the lungs vary inversely with those of the blood. It seemed reasonable, therefore, to study the effects of cooling and warming on the peripheral blood flow and the vital capacity of human subjects.

METHODS

Subjects. Five healthy men aged 21–34 years took part in the experiment. All were trained to do research work, and they were helpful and unemotional. They wore shorts or slacks, and a shirt with short sleeves or rolled-up sleeves.

Vital capacity measurements. A flow-meter with a large dial was used, allowing measurements of the amount of air blown through it and of the rate of blowing. This apparatus offers practically no resistance to expiration and, if blowing is done at an even rate, it is more sensitive than a floating cylinder. All subjects had previously practised using the spirometer. They were not allowed to see the dial so that the possibility of unconsciously influencing the results was minimized. Three readings were always taken to the nearest 50 ml. at 1½ min. intervals and averaged. Triplicate readings generally agreed to within 50 ml. and always to within 150 ml. The spirometer was kept at room temperature and carried into the cold-room or hot-room when measurements were made. The spirometer temperature was read from a mercury thermometer built into the apparatus and it remained within ± 1° C. in any one test. With a vital capacity of 5·1 and at a barometric pressure of 760 mm. Hg a rise of temperature from 15 to 16° C. would decrease the volume of water vapour condensed in the apparatus by 6 ml. Small changes of the spirometer temperature would, therefore, not have given rise to false readings. Measurements of the vital capacity were made in preference to those of the total air capacity of the lungs because the greater experimental error of the latter (Glaser & McMichael, 1941) counteracts some of its advantages. Moreover, apparatus for the estimation of the total amount of air in the lungs cannot be easily carried from one place to another, and it is sensitive to fluctuations of the environmental temperature (McMichael, 1939).

Skin and rectal temperature measurements. The skin temperature was measured with a specially designed thermocouple and portable potentiometer allowing true readings to the nearest 0·1° C. (Glaser, 1949). The rectal temperatures were taken by clinical thermometers inserted for 5 min. with the precautions previously described (Glaser, 1949). Three marked points were chosen for skin-temperature measurements, one on the radial edge of the left forearm 1 in. below the cubital fold, one near the processus styloideus radii, and one on the tip of the left middle finger. Measurements of one point on the hand give adequate information of the type, though not necessarily the extent, of vasomotor reactions happening in the extremities when the body is cooled or warmed (Glaser, 1949), but three points were chosen so that the average skin temperature of the forearm and hand could be measured.

Forearm and hand-volume measurements. These were made by simple estimations of water-displacement. A 10 ml. graduated glass pipette was mounted alongside a brass cylinder in such a way that the bottom of the pipette and the side of the cylinder communicated through a brass U-tube. The cylinder was 21 in. high and had a diameter of 5 in. It was fitted with a plumb line and rested on an adjustable tripod, so that the pipette and cylinder could always be made vertical. The zero line of the pipette was just below the upper edge of the cylinder and the 10 ml. mark was about one-third of the way down. Measurements were made by immersing the left arm into the cylinder until a horizontal line drawn across the upper edge of the olecranon was exactly in line with the upper edge of the cylinder. The water temperature was made equal to the average temperature of the forearm and hand. The water level was adjusted by pouring water in from the top or removing it through a small tap at the bottom until it corresponded to the zero line on the pipette. The arm was then withdrawn and most of the water sticking to it was shaken back into the cylinder. The volume of the arm was calculated from the reading on the pipette. The apparatus was calibrated so that 1 ml. on the pipette corresponded to 200 ml. in the cylinder. It was easy to take readings to the nearest 0·05 ml. on the pipette, which corresponded to 10 ml. in the cylinder. With practice it was possible to make an estimation without leaving the arm in the cylinder for more than 15–30 sec., even though care had to be taken not to introduce errors either by pulling the upper arm skin and thus displacing the line drawn across the olecranon or by immersing the arm diagonally. Care was taken to avoid parallax errors.

This method seemed to be more satisfactory than a plethysmograph because the possible sources of error were easily controlled and such factors as the line across the olecranon or the amount of water sticking to the forearm after its withdrawal from the cylinder were constant throughout each test, whereas in an experiment involving changes of location and very great variations of environmental and skin temperature, a plethysmograph might have given rise to many technical errors (Barcroft & Edholm, 1943). The method used, moreover, had a scope and accuracy which was similar to that of the vital capacity measurements with which it was to be compared.

Air-conditioning. There was no single laboratory in which all tests could be performed. The temperature in the hot-room was 105° F. (wet bulb) and 95° F. (dry bulb), i.e. about 40·5 and 35·0° C. and there was an air movement of about 100 ft./min. The cold-room was at +1° C. with an intermittent air-movement of about 300 ft./min. Both rooms maintained their temperature with great constancy. They were in different buildings, but measurements taken before and after cooling or warming were taken in ordinary laboratories near each air-conditioned room (some twenty paces away). The laboratory temperature varied from 14 to 21° C. but never fluctuated by more than 2·5° C. in any one test. Owing to these day-to-day changes, however, the extent of cooling or warming to which the different subjects were submitted in similar tests was somewhat variable.

Procedure. The tests were spread over 7 months between July 1947 and February 1948. Each subject was tested three times, once at room temperature and in the hot-room, once at room temperature and in the cold-room and once at room temperature only. The subjects were tested one at a time and different tests on any one subject were performed in random order and at random intervals ranging from 2 days to 3 months, so that accidental effects of climate were cancelled out. All subjects were always tested at the same time of the day, so that accidental effects of daily rhythmical variations were excluded. About 1 hr. before each test they had a light meal containing small amounts of fluid and no caffeine. During the tests they sat still on a wooden chair with their left forearm and hand resting horizontally on a padded stool. The subjects' right hand was free to adjust the mouthpiece of the spirometer, insert the rectal thermometer, or hold a book; its movements, however, were reduced to the necessary minimum. After a period of adjustment at room temperature lasting 45–60 min. the first set of measurements was taken. Fifteen minutes after these had begun the subject went to the cold-room or hot-room and stayed there for 2 hr. He then returned to where the initial readings had been taken and remained there for another 1 hr. 30 min. Measurements were made at 30 min. intervals throughout. Thus the first reading in the air-conditioned room was taken at 15 min., and the last 1 hr. 45 min. after entering it; and the first measurements after returning to laboratory temperature were made 15 min. after leaving the air-conditioned room. Control experiments at room temperature lasted about 3 hr. A period of adjustment of 45–60 min. was again allowed, and readings were taken at 30 min. intervals. Each set of measurements lasted about 6–8 min. The order of measurements was always the same. Limb-volume measurements were always taken last and the arm immediately dried so that any effects which moisture or movement may have had on the skin temperature and arm volume would have worn off before the next set of readings. The forearm was moved from a horizontal to a vertical position whenever its volume was being measured, but significant postural changes of the limb volume only set in after several minutes (Waterfield, 1931).

RESULTS

The principal findings are summarized in Table 1. A typical record is given in Fig. 1. Times given are those at the beginning of each set of measurements.

At the end of cooling the vital capacity of all subjects had fallen by 200–480 ml., the forearm and hand volume by 50–160 ml. and the average skin temperature of three points on the forearm and hand by 8–23·1° C.; after

1 hr. 15 min. at laboratory temperature the vital capacity had risen again by 270–480 ml., the forearm and hand volume by 30–130 ml. and the skin temperature by 7·7–12° C. At the end of warming the vital capacity of all subjects had risen by 180–540 ml., the forearm and hand volume by 60–130 ml. and the average skin temperature of three points on the forearm and hand by 1·8–16·5° C.; after 1 hr. 15 min. at laboratory temperature the vital capacity of all subjects had fallen again by 100–300 ml., the forearm and hand volume by 70–150 ml. and the average skin temperature by 1·9–15·0° C. In control tests at room temperature the vital capacity fluctuated by up to 180 ml., the forearm and hand volume by up to 20 ml. and the average skin temperature of three points of the forearm and hand by up to 3·0° C.

Table 1. Changes of the vital capacity, forearm and hand volume, and skin temperature on cooling, and warming

	Cooling			Warming		
Subject	Vital capacity (l.)	Skin temp. (° C.)	Forearm and hand vol. (l.)	Vital capacity (l.)	Skin temp. (° C.)	Forearm and hand vol. (l.)
	Before entering cold-room			Before entering hot-room		
G.	5·20	30·0	1·55	5·28	33·6	1·52
J.	4·90	33·5	1·40	5·12	33·2	1·26
H.	5·38	32·8	1·61	5·60	28·8	1·59
N.	4·85	25·3	1·32	4·88	20·5	1·30
T.	4·80	32·3	1·36	5·10	26·0	1·11
	After 1 hr. 45 min. in cold-room			After 1 hr. 45 min. in hot-room		
G.	4·85	22·0	1·39	5·80	35·4	1·65
J.	4·42	10·4	1·35	5·30	38·7	1·38
H.	5·18	15·7	1·54	6·00	37·2	1·70
N.	4·68	10·5	1·26	5·42	37·0	1·36
T.	4·55	14·4	1·28	5·32	35·2	1·19
	After 1 hr. 15 min. at room temperature			After 1 hr. 15 min. at room temperature		
G.	5·20	30·0	1·52	5·60	33·3	1·56
J.	4·90	21·0	1·41	5·20	33·0	1·29
H.	5·55	23·4	1·60	5·70	23·5	1·55
N.	4·95	22·5	1·30	5·18	22·0	1·29
T.	4·83	22·4	1·31	5·22	23·7	1·08

Two subjects showed an initial rise of the rectal temperature in the cold-room, followed by a fall. In one of these the rectal temperature was still 0·05° C. above the initial level after 1 hr. 45 min., but in the other it had fallen by 0·4–0·8° C. After leaving the cold-room the rectal temperature of all subjects continued to fall and finally it was 0·75–0·95° C. below its initial level in four subjects and 0·3° C. below it in the fifth. During the last 30 min. at laboratory temperature the rectal temperature of four subjects fluctuated by 0·05° C. or less, while that of the fifth fell by 0·15° C. On entering the hot-room there was a slight initial fall of the rectal temperature in four subjects, but at the end of warming the rectal temperature of all subjects had risen by 0·45–0·95° C. On leaving the hot-room there was a slight rise of the rectal temperature in one subject, but after 1 hr. 15 min. it had fallen to the initial level or to within 0·1° C. from it in all subjects.

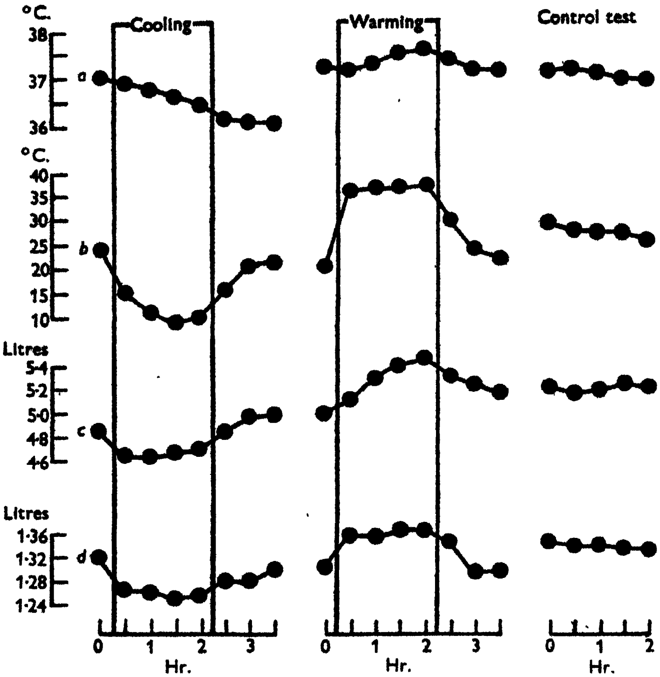

Fig. 1. The effects of cooling and warming. Typical result. Subject N. (average skin temperature of three points on forearm and hand). *a*, rectal temp.; *b*, skin temp.; *c*, vital capacity; *d*, forearm and hand volume.

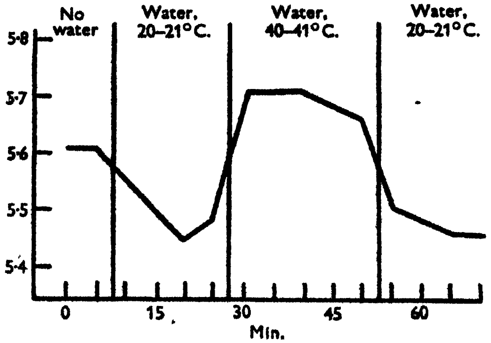

Fig. 2. The effects of cooling and warming of the lower limbs on the vital capacity. Subject G.

The effect of localized cooling or warming on the vital capacity. In order to find out whether cooling or warming of a part of the body would also result in changes of the vital capacity the following test was performed on one of the subjects. He sat still in an empty bath-tub at a room temperature of 22° C. (±1° C.) for 1 hr. Then water at 20–21° C. was quickly let in to the tub so that it covered the subject's lower limbs and buttocks up to the groin, but not his abdomen and chest. After 20 min. the water was quickly let out and the tub filled to the same level with water at 40–41° C. After 25 min. the water was quickly changed again to 20–21° C. The water was continuously stirred and its temperature kept constant. The vital capacity was measured at 5 min. intervals, taking duplicate readings each time. Cooling resulted in a moderate fall of the vital capacity and warming in a moderate rise (Fig. 2). Changes in the bath temperature were small, but the discomfort of greater changes might have interfered with even breathing. Another subject, aged 20, who had not taken part in the tests reported above, was submitted to a similar experiment. This time, however, warm water at 40° C. was first let in, and this was followed by a rise of the vital capacity amounting to 150 ml. Then the water was changed to 20° C. and this was not followed by any change of the vital capacity. Finally the water was changed to 40° C. and there was a further rise of 150 ml. in the vital capacity. When the water was removed the vital capacity fell to near its initial level. Two further subjects showed a rise of the vital capacity when their legs were immersed to the knees in water of about 40° C. and a fall of the vital capacity when their legs were immersed to the knees in water of 20° C.

DISCUSSION

In the above tests cooling was followed by a fall of the vital capacity, and the volume and temperature of the forearm and hand, while warming was followed by a rise of all these. On returning to normal laboratory temperature all these readings returned to their initial level or moved towards it by values which were greater than the experimental error. Changes of the vital capacity obtained at constant room temperature when only part of the body was cooled or warmed suggest that similar findings in the air-conditioned rooms were not caused by accidental environmental effects. The rectal temperature followed its own course which was not correlated with the other measurements, but which conformed closely with previous observations (Glaser, 1949). Fluctuations recorded in control tests at room temperature would appear to have been comparatively great, but it must be remembered that lightly clothed men tend to cool if they are sitting still at temperatures of 14–21° C. (Gagge, Winslow & Herrington, 1938) and that it may take several hours before thermal equilibrium is reached (Freeman & Linder, 1934).

It was suggested by Dastre & Morat (1884) that vasodilation in the peripheral circulation was always accompanied by vasoconstriction in the viscera. In view of the findings of Müller (1905), Müller & Siebeck (1907), Müller & Veiel (1910), Glamser (1911), Rein (1931), Sjöstrand (1935), and the results of the present investigation, a redistribution of blood between the inner organs and the periphery would seem to be one of the ways in which mammals adapt to changes of environmental temperature. It was suggested in a previous paper (Glaser, 1949) that various parts of the human body may change their temperature within comparatively wide limits, while the whole organism remains in a state of thermal balance or achieves balance at a new level. It now seems possible to expand this by suggesting that this balance is maintained by the appropriate movements of blood between the periphery (chiefly the superficial vessels of the limbs), and the organs contained inside the body cavities.

SUMMARY

1. In a cold medium the vital capacity of five subjects diminished, and so did the volume and skin temperature of their forearm and hand. In a hot humid medium all these increased. Return to normal laboratory temperature reversed these changes.

2. In four subjects immersion of the lower limbs in cold water was generally followed by a decrease of the vital capacity, and immersion in warm water by an increase.

3. It is concluded that cooling resulted in movements of blood from the extremities to the lungs, while warming caused movements in the opposite direction.

4. A simple apparatus is described, which will measure the water displacement of a limb under conditions when plethysmographic investigations are impracticable.

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THE INFLUENCE OF VARIATIONS IN THE LIGHT HISTORY OF THE EYE UPON THE COURSE OF ITS DARK ADAPTATION

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Of a variety of possible methods, one which has been used repeatedly to gain information about the adaptation process of the eye, is to study the recovery of visual sensitivity following various degrees of light adaptation (Lohmann, 1907; Rabinowitsch, 1908; Müller, 1931; Johannsen, 1934; Windsor & Clark, 1936; Hecht, Haig & Chase, 1936; Wald & Clark, 1937; Craik, 1939; Elsberg & Spotnitz, 1939; Haig, 1941; Mandelbaum & Mintz, 1941; Crawford, 1946; Clark, Johnson & Dreher, 1946; Simonson, Blankstein & Carey, 1946).

In experiments of this kind, which consist in controlling the light history of the visual mechanism prior to the dark adaptation measurements, three periods of time must be considered. First, a period before the light adaptation begins, which is here called the pre-light adaptation period; secondly, the light adaptation, which is performed under controlled laboratory conditions; and lastly, the period during which the dark adaptation measurements are made. On the basis of such experiments the view that light adaptation is completed in a few minutes is now widely held, even though Lohmann's (1907) diagram shows that light adaptation was still proceeding in his experiments some 60 min. after its commencement.

Haig (1941), for instance, states that '...light adaptation to a light of 447 ml. brightness through a 2 mm. pupil was complete in the normal observer within 4 min.' Haig's test of completeness was that prolongation of the time of light adaptation beyond 4 min., the brightness of the source remaining at 447 ml., produced no further delay in the recovery of the visual sensitivity measured after the ending of light adaptation, and that the recovery curves for the rods following 4, 6 and 10 min. of light adaptation were of constant shape and had a constant position on the graph relative to the time axis.

The object of the present experiments was to test the completeness of light adaptation (obtained by a technique similar to that used by Haig (1941)), by varying the light history of the retina in the pre-light adaptation period. If light adaptation, as obtained in Haig's experiment, was complete, then the subsequent dark adaptation measurements should be uninfluenced by adaptational changes produced during the pre-light adaptation period. In addition, the experiments have also thrown light on the mechanism of adaptation in the human eye.

METHOD

Apparatus for measuring dark adaptation. The method adopted was similar to that used by Lythgoe (1938), and a general plan of the optical system is shown in Fig. 1.

The test light field at (*G*) subtended 2° 41′ at the eyes of the subject and was displaced 7° 52′ into the right visual field by providing a small red fixation spot (*H*) at which the gaze was directed during the measurements. The intensity of this fixation light was set by the subject to be only just bright enough to achieve proper fixation.

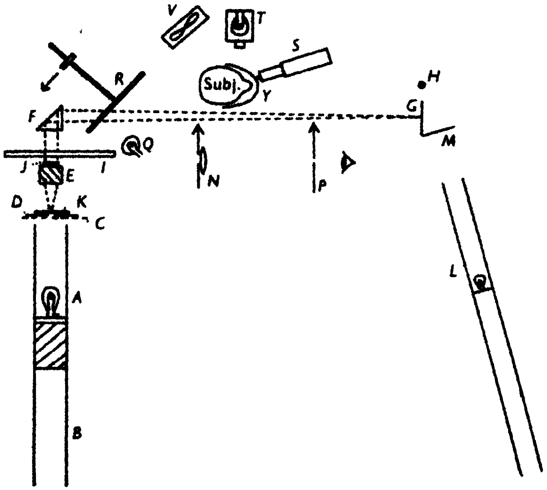

Fig. 1. Plan of the apparatus. *A*, light source; *B*, optical bench; *C*, milk glass screen; *D*, metal plate with circular aperture; *E*, projection lens; *F*, right-angled prism; *G*, white card; *H*, red fixation light; *I*, set of neutral density filters; *J*, orange 'monochromatic' Ilford spectrum filter; *K*, electromagnetic shutter; *L*, second optical bench for calibration purposes; *M*, second white card; *N*, flicker photometer lens; *P*, flicker photometer exit pupil; *Q*, lamp; *R*, whitened sector; *S*, camera; *T*, photo-flood; *V*, fan; *Y*, light adaptation screen.

A simple electromagnetic shutter (*K*) was energized approximately once every 2 sec. during dark adaptation by an impulse derived from an electrically maintained pendulum. This shutter presented the test light to the observer as a flash, the duration of which was calibrated in each series and was of the order of 0·6 sec.

Control of the voltage across the test light source (*A*) was achieved with an electronic valve circuit which reduced the normal fluctuations of the a.c. mains (±10%) to voltage fluctuations across the lamp of ±0·5%. The circuit would do this for many hours at a time without resetting.

Calibration of the test light. The calibration of the test light was undertaken in two stages. First an estimation of the brightness of the test patch with the spectrum filter removed; then the measurement of the total density of the spectrum filter. The brightness calibration was undertaken with the aid of an auxiliary optical bench (*L*). This bench was arranged to carry a flash lamp bulb. The light from this lamp illuminated another white card (*M*) of the same material as (*G*) set at an angle to the test light card so that they formed the two sides of a photometer wedge.

With this device the illumination of the test field for various positions of the source (*A*) could be equated with the illumination given by the flash lamp on the bench (*L*). The match was made at illuminations below 0·1 m.c. and thus was undertaken by rod vision. It could be reproduced to an accuracy of about ±5% even at the very lowest brightness.

A dim source of known candle-power was then substituted for the test light image and by varying its distance from the white card the test light illumination at various carriage positions and with various filters could be determined in m.c.

To convert the illumination so obtained into luminance units, the reflexion factor of the white card was determined by comparing its reflexion for white light with that from a magnesium oxide surface by means of a Holophane Lumeter. The factor was 83·7%.

An auxiliary flicker photometer was used to measure the total density of the spectrum filter. The filter reduced the light 14·12 times.

Thus the luminance of the orange test light in millilamberts (ml.) could be calculated, but in view of the complicated procedure necessary, the accuracy is probably no better than ±10%.

Apparatus for obtaining light adaptation. A curved screen of filter-paper was illuminated on the side farthest from the eyes with four 230 V. 100 W. and two 230 V. 150 W. tungsten lamps placed a few inches from the screen. The subject's eyes were placed close to this screen, which appeared under these conditions to be an evenly illuminated white surface filling the whole field of view. The luminance of the screen was measured in the first instance with a Holophane Lumeter, but since the screen lay within the near point for the subject's vision, it seemed advisable to match the apparent brightness of the screen under these close conditions with the brightness of a small white surface seen through a hole in the screen and situated well beyond the near point for distinct vision. This small auxiliary surface was illuminated by a standard lamp and its luminance was easily calculated. It appeared that 351 ml. on the auxiliary white surface was equivalent to a luminance of 312 ml. on the screen as measured by the Lumeter. All the screen luminances used were therefore converted by means of this equivalent to 'effective luminance' in ml. Subjectively, the screen appeared, on first looking at it, unpleasantly bright and light adaptation of the whole retina to luminances much in excess of those used would have to be undertaken in stages.

Photography of the pupil. In all experiments described the natural pupil was used, but in some series the area of the left pupil both during light and dark adaptation was recorded by taking a flash photograph.

Procedure. When the apparatus had been set up ready for an experimental run, the period of pre-light adaptation, the composition of which varied in each series, was undertaken by the subject (L.C.T.). Then by biting the mouthpiece the period of light adaptation was started. After every 100 sec. of light adaptation a pupil photograph was taken.

At the end of light adaptation the measurement of the threshold energy began. The change-over from light to dark adaptation, which included the removal of the light adaptation screen, took about 15 sec.

A series of test flash intensities were chosen, and as each level was 'seen' a record was made on a smoked drum with a signal marker. Immediately afterwards a pupil photograph was taken to record the area of the pupillary aperture. From the tracing on the smoked drum the time taken by the visual mechanism to reach each level in turn was obtained.

Actually the method used here is unsuitable when dark adaptation is nearly complete, because the errors of time measurement become so large. Therefore, the first 20 min. or so of dark adaptation were measured as described and then after a rest in the dark of 10 min. the 'final threshold' was measured by altering the intensity of the test light. Both time and intensity were then recorded and for the statistical treatment the errors in the time measurement were neglected. Binocular vision was used throughout for both light and dark adaptation.

The criterion. The point in time at which any test light level can be 'seen' is not precise. As the sensitivity improves during dark adaptation, an occasional flash is often seen; then later, groups of two or three flashes may be seen and be separated from similar groups by eight or ten unseen flashes. Later still, the sensitivity increases to the point where every flash is clearly seen and a decision as to what is to be accepted as the threshold sensation must be made. In these experiments observation was continued until six consecutive flashes were clearly seen.

RESULTS

In the first series of results the subject's eyes were exposed to a luminance of 482 ml. for 12 min. during the light adaptation period and the effects on the dark adaptation measurements of two types of pre-light adaptation treatment compared. Each day two experimental runs were performed: one in the early afternoon, during the last 30 min. of the pre-light adaptation period of which the subject was in a bright outdoor light, and the other, at about 10.30 p.m., which was preceded by an hour of reading in a normally lighted room, with an occasional period of darkness interspersed.

The difference in general illumination between the two treatments was of the order of 10^2, and although it might appear that a somewhat indefinite amount of pre-light adaptation had been used, it was felt that it was, at first, more appropriate to use physiological rather than laboratory conditions.

From each day's work, and for each of the chosen light levels, two times were recorded: one during the afternoon run and one in the evening. The pairs of runs were repeated seven times, and each 'evening' was subtracted from the corresponding 'afternoon' time, and the mean of the seven differences found for each chosen light level.

If, in fact, light adaptation was complete then the mean difference should on the average be zero. The values of the mean difference found are shown in the histogram of Fig. 2. Here each block represents one chosen light level and the height of the portion between the axis and the horizontal line in the middle of the shaded area gives the value in sec. of the mean difference, marked above and below the zero line according to sign.

The figures on the face of the diagram are reference numbers for the light levels, the level of highest intensity being situated on the left. Level 28 corresponds to the 'knee' of the recovery curve.

Deviations from zero of the mean difference may be due either to experimental error or to lack of completeness in the light adaptation of the eye, which has allowed the pre-light adaptation conditions to be carried over into the dark adaptation measurements. Thus the standard error of the mean difference was calculated according to the formula

$$\sqrt{[\Sigma(x-\bar{x})^2/n(n-1)]},$$

where x is any individual difference, $\bar{x}$ the mean difference, and n the number of differences. The significance of any mean difference was judged by the use of Student's distribution of t.

The standard error of the mean difference is shown in Fig. 2 as the shaded area above and below the mean itself, and the significance, for a probability level of 1 in 20, is indicated by the small sign below each block of the histogram. The test, as applied, is somewhat over-severe, since the significance of each mean difference is assessed without reference to its neighbours.

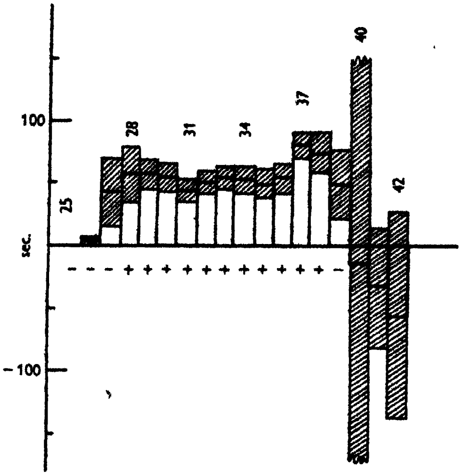

Fig. 2. The mean differences between runs performed after daylight or darkness in the pre-light adaptation period. For explanation of symbols see text. 'Evening' times have been subtracted from 'afternoon'. The figures on the face of the diagram are reference numbers for the chosen light levels. Level 28 corresponds to the 'knee' of the recovery curve.

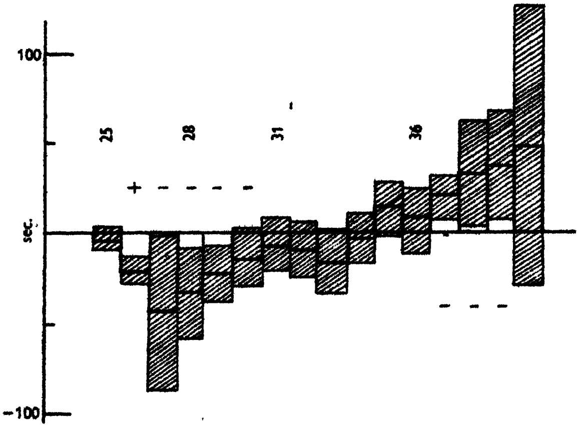

Fig. 3. A comparison between the results following 6 and 12 min. light adaptation. '12 min.' times have been subtracted from '6 min.'

Further series of runs were also undertaken in which measurements were all performed in the early afternoon, and the variation in pre-light adaptation treatment obtained either by wearing neutral 2·00 density goggles and comparing the effects so obtained with those following daylight or by the subject remaining in complete darkness for 45 min. and making a similar comparison. Some measurements were also made after light adaptation to a similar brightness but lasting for 15 min.

In all but one of these series significant differences were found in the dark adaptation measurements which were produced by alterations in the pre-light adaptation history of the eye, and thus the conclusion must be drawn that adaptation to a brightness of 482 ml. for 12 or 15 min. leaves the visual mechanism incompletely light adapted.

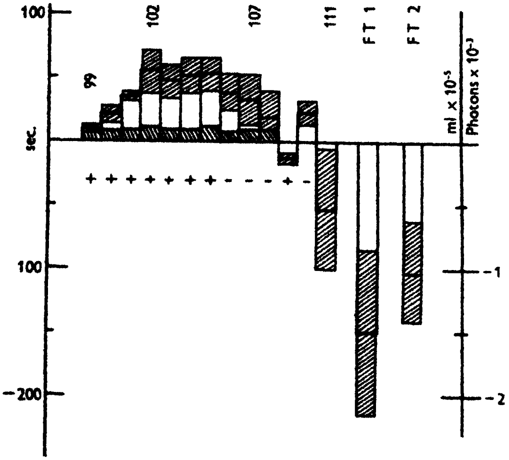

Fig. 4. A further comparison during which the pupil diameter was determined by photography. 'Dark' times have been subtracted from 'light'. Level 101 corresponds to the 'knee' of the recovery curve.

That completeness of light adaptation as judged by Haig's (1941) criteria has been achieved, may be seen from Fig. 3. Here a comparison has been made between runs in which the light adaptation to a brightness of 482 ml. was either for 6 or 12 min., the pre-light adaptation treatment being similar in each run. The differences could be due to the experimental error. Thus prolongation of the light adaptation from 6 to 12 min. caused no further delay in recovery, so that 6 min. light adaptation to a brightness of 482 ml. would normally be considered complete.

One criticism of the above results might be that owing to the use of the natural pupil the significant mean differences were produced by appropriate alterations in pupil diameter, leading to differences in retinal illumination. To meet this criticism a series was performed in which the diameter of the left pupil was measured throughout by pupil photography. Light adaptation was to a brightness of 494 ml. for 15 min., and pre-light adaptation was either 45 min. in daylight or a similar time in darkness. Fig. 4 shows that the usual significant mean differences were again found. These differences *were* associated with significant differences in pupil diameter, which were in such a direction that they could have accounted for the observed threshold differences on the basis given above. However, when the retinal illumination was calculated for both 'light' and 'dark' runs (due allowances being made for the Stiles-Crawford effect (1932)), the interesting fact emerged that the pupillary changes, although in the right direction, were of insufficient magnitude to account for the whole of the significant threshold difference found. The small cross-hatched pieces at the foot of the blocks in Fig. 4 give the amounts of the threshold differences which could be explained by pupillary change.

DISCUSSION

Most modern workers have regarded light adaptation as a process which is over in a few minutes, none having tested the completeness of light adaptation by varying the pre-light adaptation history. The results of the present series of experiments do not support the view that light adaptation is a rapidly completed process. Equilibrium in the visual mechanism is only achieved by light adaptation for considerable periods of a similar order of magnitude to those required for the completion of dark adaptation.

The idea that light adaptation is a slow process is not new. Lohmann's (1907) results also agree with this view. Lohmann's work has been criticized by Wald & Clark (1937), and it is true that his method of threshold determination is not very sound.

That the visual apparatus does not reach a steady state even after 24 hr. in the dark has been shown by Achmatov (1926). This author's work is supported to some extent by that of Semeonoff (1941), who followed dark adaptation for several hours. The effects of light adaptation also appear to be very longstanding in some cases.

Clark *et al.* (1946) found that the light adaptation of the visual mechanism obtained by several hours on the beach in Florida under full sunlight could be detected in threshold observations made on the following day, after a night's rest.

It may well be possible only to obtain an approximately steady adaptational state, and slow increases or decreases of sensitivity may continue for many hours after any change in light conditions.

The mechanism of adaptation. The persistence of the effect of pre-light adaptation might lie either in the photochemical system, in the receptors, in the retinal or lateral geniculate synapses or in the brain, but the work of Craik & Vernon (1941) on an eye blinded by pressure makes it probable that the effect lies within the eye itself. If this is so, the observed threshold differences described above may be due, if pupillary differences do not account for them, either (*a*) to the failure of the light during the 15 min. adaptation to bring the photochemical system of retina to a steady light adapted state; or (*b*) to pre-light adaptation effects persisting in the nervous apparatus of the eye.

If the first is correct, differences in the concentration of the photosensitive substances or their photoproducts have suitably modified the pupil size, *whilst adaptation is proceeding in darkness*. This might be possible either through the medium of thermal or photochemical decomposition.

Lythgoe & Quilliam (1938) have investigated the thermal decomposition of visual purple in solution and have found that the speed of such decomposition varied with both the temperature and pH of the solution. Thermal decomposition was slowest for pH values around neutrality. At 37·5° C. it was only just measurable after 30 min. from the beginning of the experiment. Dartnall, Goodeve & Lythgoe (1938), whilst measuring the effect of temperature on the photochemical decomposition of visual purple, made allowances in their measurements for thermal decomposition. These allowances at pH 7·8 and temperature 35·0° C. were less than 1% of the measurements. Thus it seems unlikely that visual purple could influence the sensory message in darkness by thermal decomposition.

Could the photochemical decomposition associated with the perception of the test flash be the means by which differences of concentration of photosensitive material are transmitted to the iris muscles during dark adaptation?

Since the sensory message from that portion of the retina stimulated by the flash is a threshold message in runs following all types of pre-light adaptation treatment, the message reaching consciousness must be of similar magnitude in each case. Thus, unless one adopts the idea (for which there is no evidence) that the pupil is controlled by a sensory message different from that on which the sensations are based, differences in photochemical concentrations cannot, via photochemical decomposition, affect the measurements of pupillary diameter when the eye is in darkness.

The first hypothesis is thus unsatisfactory because it appears to be impossible for the photochemical system of the eye to influence the pupil size in the dark, and so lead to the significant differences of pupil diameter found in the experiment. If the nervous system could play some part in adaptation the differences reported here could be easily explained as residual differences in nervous adaptation which are not fully obliterated by light adaptation. It is well known (Granit, 1947, for instance), from electrophysiological experiments, that many visual receptors continue to discharge at a slow rate even when not illuminated and differences in adaptation could be manifest by these discharges and signalled to the iris muscles *even though the eye is in darkness*.

Whilst these experiments may provide evidence for a nervous component to visual adaptation they unfortunately give no indication as to whether this component lies within the receptors themselves or in the retinal synapses.

SUMMARY

1. Measurements of the recovery of visual sensitivity following light adaptation under controlled conditions have been performed by a threshold method.

2. Light adaptation to a luminance of 482 ml. for 15 min. has been shown to be incomplete by noting the effect upon the threshold measurements of variations in the light history of the retina prior to light adaptation.

3. Differences of pupil diameter following two different pre-light adaptation treatments are best explained by postulating a nervous component to visual adaptation.

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VASCULAR REACTIONS IN THE HUMAN INTESTINE

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Changes in blood flow will alter the temperature of animal tissue only if the tissue concerned is exposed to an environmental temperature different from that of the blood. If the blood and the environment each remain at constant temperature it can be assumed that the temperature ultimately reached by the tissue will depend upon the rate of flow of the blood. Parmenter & Benedict (1929) used skin temperatures in this manner as an index of cutaneous blood flow reactions. Barcroft & Millen (1939) similarly used the deep temperature of the calf to demonstrate circulatory changes in human muscle.

In the present investigation, temperature changes have been used to demonstrate vascular responses in the human intestine, mainly in response to body warming, the experimental material consisting of intestinal tissue exposed on the surface of the abdomen following the operations of colostomy, caecostomy or ileostomy (Grayson, 1949*a*).

METHODS

The thermoelectric apparatus. The circuit comprised cold junction, external resistance, moving coil mirror galvanometer and a connexion box whereby any one of six thermocouples could be quickly switched into the circuit.

For the measurement of colonic tissue temperatures fine needle thermocouples, made from no. 20 hypodermic needles, with 40 s.w.g. silk covered constantan and 40 s.w.g. enamelled copper wire threaded down the bore and soldered at the tip, were used. Fine loop thermocouples (Grayson, 1949*b*) were used to measure skin temperatures from the fingers. The temperatures of the abdominal skin and of the room were also measured electrically.

All leads were of standard lengths. Even so, slight variations in resistance were found between individual thermocouples. In order to minimize the differences in readings which might consequently result, a high resistance circuit was used. The galvanometer had an internal resistance of 200 Ω. and a sensitivity such as to give a deflexion of 1 mm./μV. with the scale at 1 m. With the scale at 2 m. and with the apparatus calibrated to show temperature differences of 0·02° C., the external resistance required was about 800 Ω. Under these conditions the variations in resistance between thermocouples were found to be insignificant.

The measurement of intestinal temperature. Patients were selected on whom colostomies, caecostomies or ileostomies had been performed in the treatment of neoplastic or inflammatory conditions of the large bowel or rectum. In all cases the exposed tissue was healthy and the patient afebrile. In two cases, where loops of intact bowel had been brought to the surface in readiness for opening later, the experiments were performed 2–3 days after the first operation. In the other cases, where the colostomy was open and functioning normally, the experiments were performed 1–6 months after operation.

All measurements were made in a temperature-controlled room from which draughts were excluded and where the temperature was normally 20·0 ± 0·25° C. Rectal and skin thermocouples were adjusted and the needle thermocouples inserted into the muscular coat of the exposed bowel.

Temperature distribution in the exposed bowel. Where small amounts of intestinal tissue were exposed there were only slight temperature variations between different parts of the exposed bowel wall. In bulky colostomies, however, there were differences of up to 1·5° C. according to the position of the needle in relation (*a*) to its depth below the surface, and (*b*) to its distance along the axis of the colostomy from the point where the bowel emerged through the abdominal wall.

It was found that depth below the surface was relatively less important than distance along the axis. In one patient, for example, examined 5 days after operation when a considerable amount of colonic tissue was still evident, it was found that near the base of the colostomy the subserous temperature was 30·9° C., whereas about $\frac{1}{4}$ in. below the surface, in the deep muscle coat, the temperature was 31·3° C. In the same patient the temperature of the deep muscle coat at the apex of the colostomy, approximately $\frac{1}{2}$ in. distal to the base, was 29·8° C. In view of these and other similar observations all experiments were carried out with the needles inserted near the base of the colostomy in a plane parallel with the abdominal wall, so that any slight movement of the needle would take place in the plane of least pronounced temperature gradient.

RESULTS

Temperature changes in bowel exposed to room air

On removing the dressing from a colostomy, two distinct phases could be distinguished in the tissue temperature reactions.

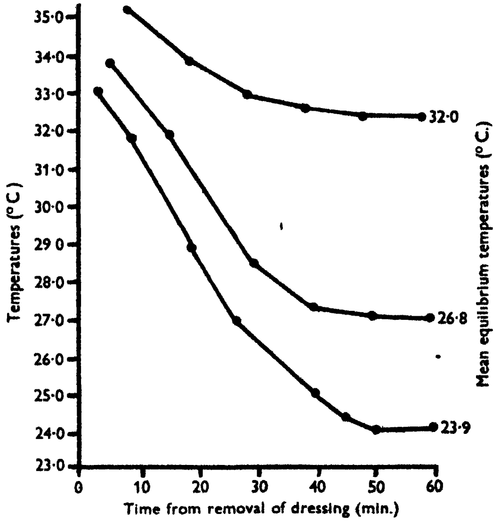

Fig. 1. Rate of cooling in a colostomy after removing the dressing—three different cases.

The phase of cooling. On first exposure to room air a drop in temperature was always observed. The initial temperature varied with the delay in beginning readings, but was usually between 33·0 and 36·0° C. An equilibrium temperature higher than that of the room was reached after 45–50 min. (figure 1).

The phase of equilibrium. Four control experiments (Fig. 2) were performed in which the temperature of the bowel was allowed to fall to equilibrium and readings continued for further periods of up to 2 hr. The room temperature remained constant at 20·0° C. and there were no significant fluctuations in rectal temperature. Under these conditions it was found that the equilibrium temperature, once reached, was maintained for the duration of the observations.

In an additional sixteen experiments (Table 1) the equilibrium temperature was observed over periods of about 20 min. It will be seen that it varied between 28·8 and 35·5° C., the average being 32·5° C.

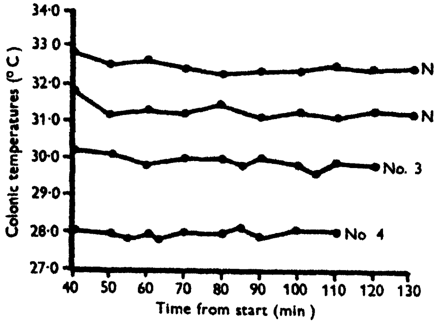

Fig. 2.

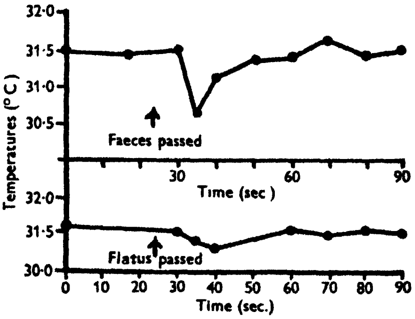

Fig. 3.

Fig. 2. Control experiments. Initial cooling curves omitted.

Fig. 3. Effect of bowel action on colostomy temperature. Upper record: passage of faeces. Lower record: passage of flatus.

Changes in the equilibrium temperature under constant conditions

Transient fluctuations in equilibrium temperature were observed as a result of bowel action and, occasionally, without obvious cause.

Bowel action and the equilibrium temperature. Passage of flatus always caused a small drop in colostomy temperature, usually less than 0·3° C., appearing after a latent period of about 20 sec. The bowel temperature returned to its base line within 2 min. of the first appearance of fluctuation (Fig. 3).

Passage of faeces rarely occurred during experiments on colostomies of the descending colon but was more common with colostomies of the transverse colon, caecostomies, or ileostomies. There was a latent period of up to 20 sec. after the faeces began to pass before the temperature of the bowel began to fall. The maximum drop recorded from these causes was 0·8° C. The original equilibrium was regained within 2 min. after the passage of the motion.

Spontaneous fluctuations. Spontaneous changes in bowel temperature were rare. Occasionally, however, without obvious cause, a transient increase in bowel temperature occurred, accompanied by a decrease in skin temperature. In the case recorded in Fig. 4, draughts, bowel action and outside disturbances were excluded. The patient was quiet and the rectal temperature did not vary. Two fluctuations occurred in a period of 2 hr., increases in bowel temperature of 0·7 and 1·0° C. being accompanied by reductions in skin temperature of 1·2 and 0·9° C. Each effect lasted about 5 min. and was followed by a return to the base-line.

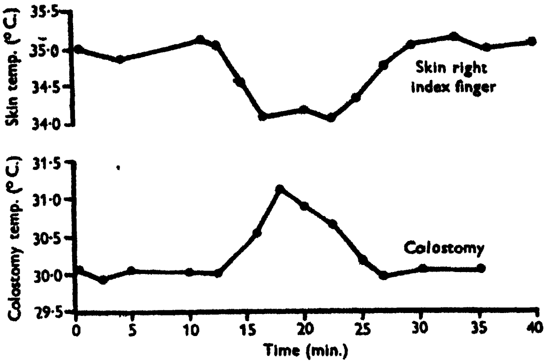

Fig. 4. Spontaneous fluctuations simultaneously recorded from the skin of the index finger and from the colostomy.

The effect of body heating on the temperature of exposed bowel

In each of the following experiments the bowel was left exposed to cool to an equilibrium, and a further 20 min. allowed to establish the base-line. Body heating was carried out by wrapping the subject in blankets, applying hot-water bottles and immersing one limb in water at about 43° C., the colostomy being left exposed. Temperature records were taken from the colostomy wall, from the skin of the left index finger and, where possible, from the rectum. In some patients in whom the rectum had been resected, temperatures were taken instead from high up the colon.

The results of sixteen experiments are given in Table 1. A typical result is shown in Fig. 5.

Two phases could be distinguished in the temperature reactions of the exposed bowel, whether colon, caecum or ileum, namely (*a*) a phase of falling bowel temperature and rising skin temperature, and (*b*) a phase of rising bowel temperature seen after prolonged heating.

The phase of falling bowel temperature. The bowel temperature began to fall after a latent period of 5–15 min., reaching its lowest point after 20–50 min. There was a considerable variation in the extent of the cooling. The maximum recorded drop during a heating experiment was 4·5° C.; the least recorded drop was 0·9° C., the average being 2·8° C.

Skin temperatures from the left index finger rose at the same time. Maximum levels of about 35° C. were reached after 20–50 min. of body warming.

Rectal temperatures showed no change during this early phase of the experiment.

TABLE 1. Effect of body warming on the temperature (° C.) of a colostomy allowed to cool to equilibrium by exposure to room air at 20·0° C.

	Duration of body heating in (min.)								Rectal temp. (° C.)		
Type of colostomy	0	10	20	30	40	50	70	90	0	40	90
Descending colon; open	30·8	28·6	27·3	27·1	27·0	—	—	28·7	36·7	36·7	37·2
	31·0	30·8	28·5	26·9	26·5	26·6	26·5	27·2	37·0	37·1	37·3
	35·5	35·0	34·3	34·2	33·9	34·0	35·0	—	37·2	37·0	37·2
	34·2	33·2	32·5	32·1	31·8	31·9	33·0	—	38·2	38·2	38·6
	33·5	31·6	29·9	29·0	30·6	31·2	31·5	32·0	37·0	36·6	37·1
	31·2	30·7	30·1	28·8	28·3	27·0	27·6	27·9	37·1	36·9	37·2
	29·0	29·0	28·0	27·0	27·3	27·5	28·0	—	36·8	36·8	37·0
	32·0	30·0	29·3	28·9	28·5	29·0	29·5	29·8	37·6	37·5	38·3
	35·5	34·8	34·3	33·8	34·4	34·5	—	—	37·8	37·8	38·1
Transverse colon; open	32·0	31·0	29·8	29·2	30·0	30·5	31·2	—	37·3	37·1	37·3
	33·2	32·9	32·7	32·4	32·2	31·9	31·9	—	36·9	36·9	37·2
Caecostomy; open	30·0	28·9	28·5	28·2	28·0	28·5	28·8	30·0	37·5	37·7	38·5
	35·3	35·2	34·3	34·6	—	34·7	—	—	37·1	37·1	—
Terminal colostomy of descending colon; open	33·0	32·4	31·1	30·2	30·1	29·4	29·0	—	37·5	37·0	36·9
	28·8	28·4	27·3	27·1	—	27·3	—	—	37·2	37·1	—
Unopened colostomy of descending colon	34·5	33·7	32·2	31·6	32·0	32·5	32·7	—	37·3	37·1	37·4

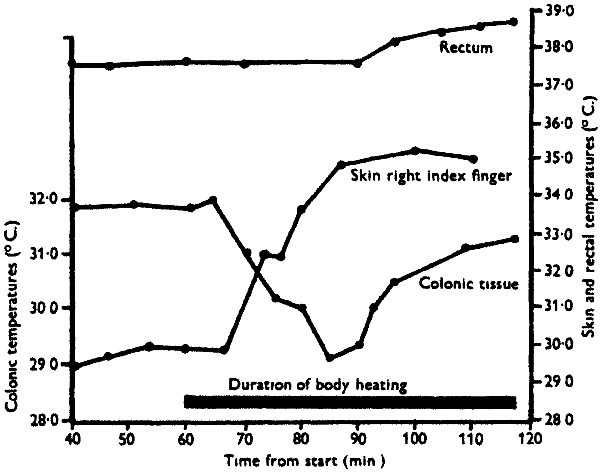

Fig. 5. The effect of body heating on colostomy, skin and rectal temperatures—initial cooling curves omitted.

The phase of rising bowel temperature. In most cases the early drop in bowel temperature was followed by a rise, beginning 30–60 min. after the start of body heating (Fig. 6). It was accompanied by the onset of perspiration and by an increase in the rectal temperature. The rise in colonic tissue temperature varied between 0·7 and 3·0° C. In six cases there was a rise in colostomy temperature of 2·0° C. or over with corresponding increases in rectal temperature of from 0·2 to 0·6° C. The skin temperature of the index finger was usually at a maximum before the bowel temperature began to rise.

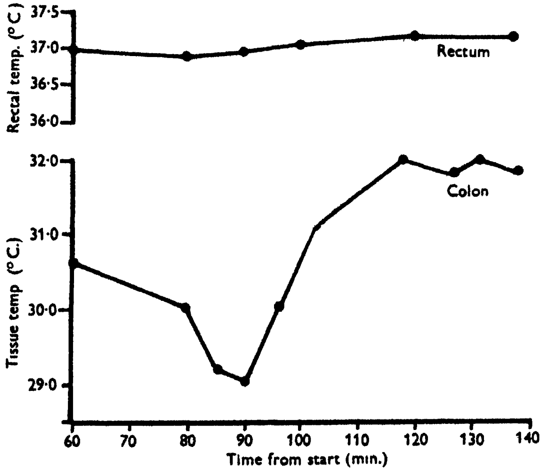

Fig. 6. The effect of prolonged body heating on the colonic temperature.

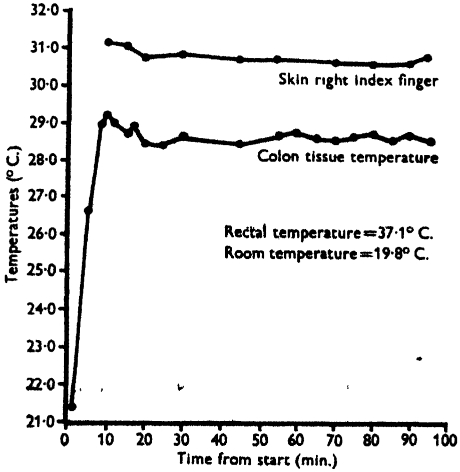

Fig. 7. Control experiment on colostomy previously cooled by means of ice pack. Pack removed just before start of observations.

Experiments on cooled colostomies. These experiments were performed (*a*) to supply additional evidence showing that the drop in bowel temperature which followed body heating bore no relation to the initial cooling of the colostomy, and (*b*), by using a procedure which did not involve long periods of stabilization, to shorten the experiments and lessen the strain on the patients.

In a typical experiment the colostomy was packed with ice at the start so that its temperature fell to below that of the room. When the ice was removed the colonic temperature rose rapidly. In a control experiment (Fig. 7) in which no body heating was carried out and the patient lay quietly in the temperature-controlled room, an equilibrium temperature of about 28·5° C. was reached and subsequently maintained for the period of the observations.

TABLE 2. Effect of body warming on the temperature (° C.) of colostomies exposed to the room air after previously cooling with ice and allowing to warm to an equilibrium

	Duration of body heating in (min.)								Rectal temp. (° C.)		
Type of colostomy	0	10	20	30	40	50	70	90	0	40	90
Transverse colon; open	33·5	32·2	31·1	30·6	29·8	29·4	28·8	30·0	37·3	37·1	37·6
Descending colon; open	32·6	32·3	32·2	31·5	30·0	28·1	27·8	28·8	37·1	37·0	37·3
Descending colon; open	33·1	32·4	31·1	30·3	30·9	31·2	—	—	37·4	37·3	—
Descending colon; unopened	22·5	22·0	21·6	22·5	23·6	23·8	—	—	37·5	37·1	37·4
Ileostomy; open	35·6	35·6	35·3	34·2	34·3	33·1	32·5	34·5	36·9	36·9	37·2
Caecostomy; open	35·0	35·0	34·9	33·9	35·0	36·5	—	—	37·5	37·3	37·6

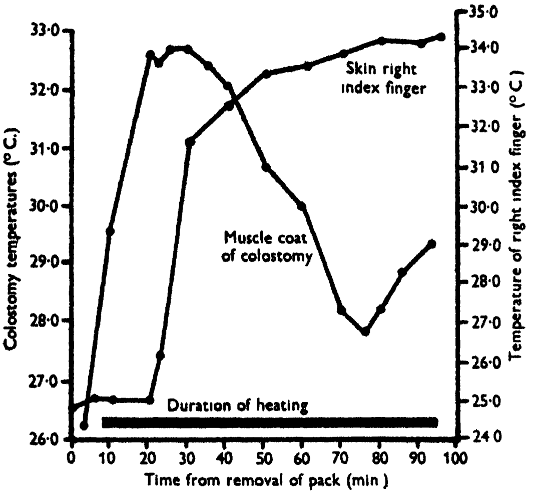

Fig. 8. The effect of body heating on colostomy previously cooled by means of ice pack. Same case as Fig. 7.

In six other experiments (Table 2), the tissue after cooling was allowed to warm towards equilibrium, when body heating was carried out as in previous experiments. The result shown in Fig. 8 was obtained with the patient used in the control experiment (Fig. 7).

In each experiment body heating still produced a fall in bowel temperature, which was, however, clearly separated from the initial cooling by a phase of rising bowel temperature.

DISCUSSION

When the dressing is removed from a colostomy its temperature falls to a level, which, under conditions of quiet and constant room temperature, can be maintained for considerable periods.

The effect of heating the body was observed in two series of experiments. In the first, the dressing was removed from the colostomy which was allowed to cool by simple exposure to the room air. About 50 min. was required for cooling and a further 20 to confirm the base-line. Body warming carried out in these patients produced a marked drop in the temperature of the exposed bowel.

None the less, despite the consistency of the results, it was felt that the 20 min. stabilization period did not emphasize sufficiently the difference between this phase of cooling and the cooling which occurred initially on removing the dressing. A second series of experiments was performed in which, after removal of the dressing, the bowel was cooled rapidly by ice applications to about room temperature. On removing the ice the bowel warmed up to its equilibrium. In all these experiments, heating the body still produced a fall in bowel temperature. The room temperature was constant and there was no rise in rectal temperature. It can be concluded, therefore, that this fall in the temperature of the exposed bowel on heating the body is independent of the initial phase of cooling and is the result of a diminished blood flow in the bowel.

The experiments indicate that whereas body heating produces a vasodilatation in the skin, as long as the rectal temperature does not rise, it produces a concommitant vasoconstriction in the exposed bowel. It may also be significant that apparently spontaneous fluctuations in the skin blood flow are accompanied by opposite fluctuations in the temperature of the bowel.

In experiments where the body heating was sufficient to produce a rise in rectal temperature, the phase during which the bowel temperature fell was followed by one in which it rose. In every case the temperature increase of the exposed bowel greatly exceeded that of the rectum, so that on purely physical grounds the tissue temperature increase cannot be a simple result of the rise in blood temperature. The effect must be due to an increase in blood flow in the exposed bowel, that is to say a vasodilatation of the intestinal vessels.

SUMMARY

1. Thermoelectric methods have been used to measure the temperature of bowel wall exposed on the surface of the abdomen following the operation of colostomy, or ileostomy in human subjects.

2. Control experiments show that, under constant conditions, temperature equilibrium is maintained for long periods in the exposed bowel.

3. Spontaneous vasoconstriction in the skin of the extremities is accompanied by a spontaneous increase in the temperature of the colostomy.

4. Body heating produces a fall in the temperature of the colostomy tissue, probably the result of a vasoconstrictor response in the intestine which accompanies the vasodilator response in the extremities.

5. Body warming sufficient to produce an increase in rectal temperature is accompanied by a rise in the temperature of the colostomy, probably the result of a vasodilatation in the intestine in response to raised body temperature.

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THE RELEASE OF HISTAMINE BY D-TUBOCURARINE FROM THE ISOLATED DIAPHRAGM OF THE RAT

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Alam, Anrep, Barsoum, Talaat & Wieninger (1939) have shown that, when the dog's gastrocnemius is perfused with defibrinated blood, injections of curare into the perfusion fluid cause a release of histamine from the muscle. These findings have been confirmed by Schild & Gregory (1947).

The present communication deals with a simple diffusion method by means of which the release of histamine from striated muscle by D-tubocurarine may be demonstrated and studied quantitatively. When the rat's diaphragm, which has a relatively high histamine content, is placed in Ringer's solution containing an adequate amount of curarine, most of the histamine contained in the muscle becomes diffusible and is released into the surrounding fluid. It will be shown that, above a certain concentration of curarine, the rate at which histamine diffuses from the muscle becomes constant and is presumably only limited by the rate of diffusion of histamine through muscle. On this assumption a 'diffusion constant' of histamine through muscle has been calculated and its magnitude has been found to be of the same order as that of urea as determined by Conway & Fitzgerald (1942).

METHODS

The diffusion method. Rats weighing 200–400 g. were killed by a blow on the head and bled. The diaphragm was then dissected out, washed in cold Tyrode solution to remove traces of blood, cleaned of connective tissue and divided. Usually the two lateral portions of the diaphragm, which are similar in thickness and shape, were used as control pairs. Strips of muscle weighing 150–300 mg. were attached to platinum hooks fused into the tip of capillary glass tubes, transferred to warm Tyrode solution, and thence into the experimental solution containing D-tubocurarine dissolved in Tyrode solution at 37°, and stirred by a stream of oxygen. After a measured time the muscle was removed from the solution which was then ready for assay. The muscle may be placed in further experimental solutions until no more histamine is released. At the end of the experiment the histamine remaining in the muscle may be extracted with trichloracetic acid (Code, 1937) or by boiling with saline (Feldberg & Kellaway, 1937).

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The volume of experimental solution was usually 2–5 c.c. and the ratio of fluid to muscle weight of the order of 10 to 1. The mean thickness of the diaphragm muscle was 0·75 mm. as computed from weight and surface. The latter was estimated by drawing the contour of the flat muscle on a piece of paper and weighing the paper.

Histamine assays. The solutions were assayed without further treatment. Histamine determinations were done on the guinea-pig's ileum using a statistical method (Schild, 1942). A small organ bath of about 4 c.c. volume was used. Histamine values are expressed in terms of histamine acid phosphate/3.

Effect of curarine on histamine assay. The presence of D-tubocurarine in the solutions slightly modifies the histamine response of the guinea-pig's ileum. D-Tubocurarine itself has no effect, but it often slightly potentiates the response to a given dose of histamine. Fig. 1. shows that the addition of 0·8 mg. D-tubocurarine to 0·02 μg. histamine slightly increases the response. At other times, when larger doses were used, or when the preparation was fatigued, curarine slightly depressed the histamine response. In order to obviate this interference, the same amount of curarine as was present in the test solution was added as a rule to the histamine standards. We are indebted to the Wellcome Foundation for a supply of crystalline D-tubocurarine, which was readily soluble in Tyrode solution.

Effect of benadryl and atropine. A concentration of benadryl of 1:100 million inhibited the effect of the active substance released by D-tubocurarine quantitatively to the same extent as that of histamine (Fig. 2 A). This suggests that the released substance is either histamine or closely related to it (Schild, 1947). Atropine in a concentration sufficient to abolish the effect of acetylcholine produced a barely perceptible depression of the effects of the released substance and of histamine (Fig. 2 B).

A typical experiment is shown in Fig. 3. In this experiment the muscle was left for nine successive periods of 5 min. in solutions of 1:1000 D-tubocurarine, and a total of 13·7 μg. histamine per g. of tissue was released. In control experiments of the same duration the total amount of histamine diffusing from the muscle was of the order of 0·4 μg./g. of tissue (less than one-thirtieth). Similar changes of the bathing solution at frequent intervals were practised in the majority of the longer experiments in order to maintain the full diffusion gradient of histamine.

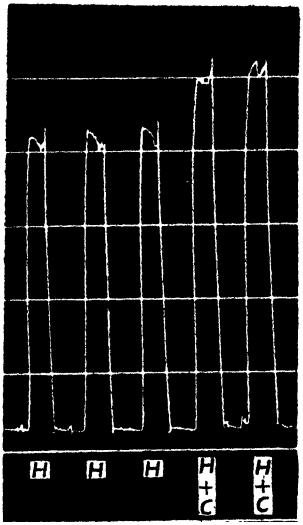

Fig. 1. Potentiation of histamine effect by D-tubocurarine. Contraction of guinea-pig ileum. H = histamine alone (0·02 μg.); $H + C$ = same dose of histamine given together with 0·8 mg. D-tubocurarine.

RESULTS

Histamine release and concentration of curarine

The concentrations of curarine which produce a release of histamine are very much higher than those required for curarization at the neuromuscular junction. This point will be discussed in detail in a subsequent paper. It is necessary to distinguish between the effect of concentration of curarine on the *rate* of histamine release and on the *total amount* released until liberation is completed. The effect on the total amount is difficult to determine since the experiments cannot be indefinitely prolonged, the effect on rate, however, is very pronounced. An example of this is shown in Fig. 4 which represents two parallel experiments in which concentrations of 1:1000 and 1:4000 curarine were used.

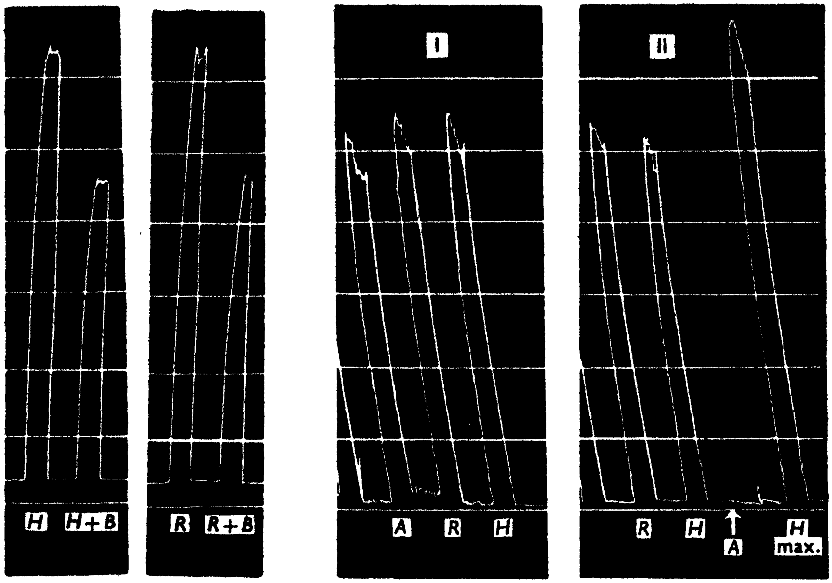

Fig. 2 A. Fig. 2 B.

Fig. 2 A. Effect of (1:100 million) benadryl (*B*) on contraction produced by histamine (*H*) and substance (*R*) released by curarine. B. Effect of (1:5 million) atropine on released substance (R), histamine (H) and acetylcholine (*A*). Between I and II atropine was added to the Tyrode solution.

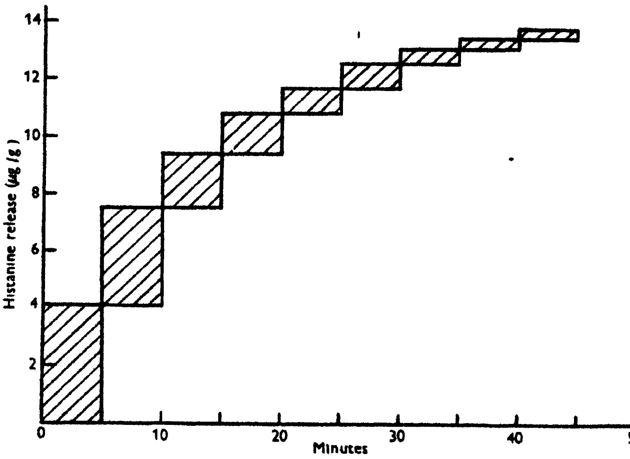

Fig. 3. Total histamine released from a diaphragm which was left during successive periods of 5 min. in solutions of D-tubocurarine (1:1000) in Tyrode solution.

In each case 0·5 g. of muscle tissue was left in 10 c.c. of solution, of which small samples were withdrawn periodically for assay. The difference is mainly one of rate and not of final amount released, e.g. the relative activity of the two solutions is 1:5 after 10 min. but only 1:1·2 after 90 min.

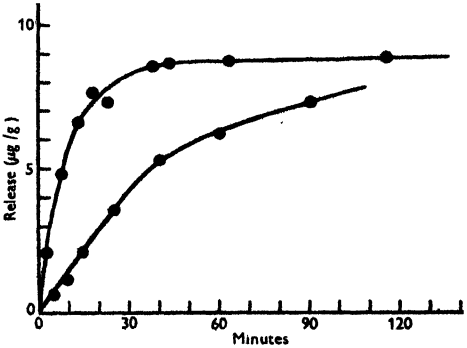

Fig. 4. Rates of release of histamine with concentrations of 1:1000 (upper curve) and 1:4000 D-tubocurarine (lower curve).

The initial rate of release. The simplest way of determining the effect of curare concentration on the rate of histamine release is to measure the initial rates. Fig. 4 shows that the time-release curves are exponential, but that during the initial stages they are approximately linear. Our main object was to find out whether the rate attained a maximum or whether it would continue to increase with increasing concentrations of curarine. Table 1 shows that the initial rates increase rapidly within the range of concentrations of 0·25–1·0 mg./c.c. curarine, but that above this range they become approximately constant. Since the greatest amount released in these experiments is less than half the amount that would be released over longer periods, it follows that the rate of release attains a limiting value beyond which a further increase in the concentration of curarine produces little or no effect. The probable reasons for this limitation are discussed in the next section.

TABLE 1. Initial rates of histamine release

Average of two experimental periods of 3 and 5 min. duration

Conc. D-tubocurarine (mg./c.c.) ...	Nil	0·25	0·5	0·75	1·0	2·0	3·0	4·0
Histamine released (μg./g.) ...	0·1	0·35	1·7	2·2	3·7	4·8	3·5	5·0

Histamine release as a diffusion process

The release of histamine from the isolated diaphragm by curarine is a complex process, the rate of which presumably depends on many factors. Two obvious limiting factors must be the rates of diffusion of curarine and histamine through muscle. Although curarine presumably diffuses more slowly than histamine, since it is a larger molecule, yet the time taken up by its diffusion may be rendered negligible by the simple device of increasing its concentration in the surrounding fluid. The greater the concentration in the surrounding fluid the less time is needed to build up an adequate concentration at the site of action. The time for diffusion of histamine cannot in the same way be made negligible,

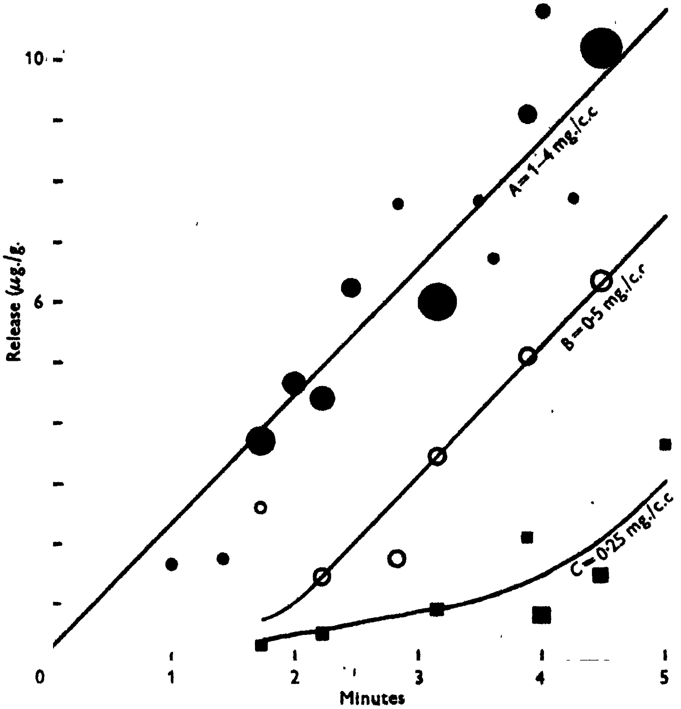

Fig. 5. Summary of 106 individual measurements of histamine release by curarine. Large circles represent mean values, the area of each circle being proportional to the number of observations. Black circles: histamine release with concentrations of curarine of 1 mg./c.c. or more; white circles: 0·5 mg./c.c.; squares: 0·25 mg./c.c. The slope of line *A* has been used for the calculation of the diffusion constant of histamine. The root mean square deviation of individual points from the regression line *A* is ±2·5 μg./g.

it must always remain a limiting factor, and we have assumed that when the concentration of curarine in the surrounding fluid is sufficiently high, histamine diffusion becomes the main release rate determining factor. This is borne out by the results shown in Fig. 5.

Fig. 5 shows the quantities of histamine released by various concentrations of curarine plotted against the square root of time. In a simple diffusion process the points should lie along a straight line passing through the origin. Regression line *A* is the mean square regression calculated from seventy-six original observations on histamine release using concentrations of curarine of 1 mg./c.c. or more. It will be seen that the regression line passes almost exactly through the origin and that the experimental points lie along a straight line. Lines *B* and *C* drawn through points corresponding to lower concentrations of curarine do not pass through the origin and have a complex shape which need not be discussed here. These results suggest that, provided the concentration of curarine is sufficiently high, histamine release can be treated as a simple diffusion process starting practically at zero time, the rate of which is determined by the rate of diffusion of histamine through muscle. An apparent diffusion coefficient of histamine through muscle may thus be calculated from the slope of regression line *A*. For this purpose, however, it is necessary first to express diffusion in terms of diffusing surface rather than in terms of weight, and to determine the diffusion head of histamine, or the concentration of diffusible histamine within the muscle.

The cencentration of diffusible histamine in the muscle

Histamine is presumably held within the cell in an indiffusible form. The clearest proof of this is found in some experiments by one of us (H.O.S., unpublished), which show that histamine when added to muscle permeates freely through tissue water. If intracellular histamine were freely dissolved in the cell water it would probably diffuse out just as easily. Bound histamine may be localized in certain parts of the cell, but when it is released it may be assumed to acquire rapidly a uniform concentration throughout the muscle. This assumption is based on the view discussed in dètail by Conway & Kane (1934) that muscle can be regarded as a uniform diffusion mass and that diffusion is conducted through the tissue elements and not along the interspaces. Otherwise histamine might conceivably diffuse into the surrounding tissue space from a high concentration head situated near the cell surface without ever attaining equilibrium within the fibres.

The quantity of histamine that can be released by curarine need not necessarily be the same as the quantity that can be extracted by chemical procedures which destroy the cell structure, it might conceivably be either more (through formation of histamine) or less. Table 2 shows that, in fact, the maximum quantity that is released by curarine is always less than the total that can be extracted from a control muscle. In this respect curarine seems to differ from ammonia which releases all histamine present in the cell (Schild, 1949). In some of these experiments the muscles were left in curare solution for periods up to 3 hr. and then extracted. In all cases an appreciable amount of histamine could be extracted at the end of the experiments, averaging 35% of the original amount present in the controls or 30% if only the experiments of longest duration are considered. The quantities released and left behind add up approximately to the original activity.

It may be argued that insufficient time was allowed for complete diffusion. It can be calculated, however, that given the initial diffusion rate (and a simple diffusion process) over 99% should have been released at the end of 3 hr. It can be concluded therefore that only about 70% of the total histamine present in muscle is released by curarine.

The apparent diffusion coefficient of histamine through muscle

This may now be estimated from the equation

$$P=2c_0\sqrt{\frac{kt}{\pi}},$$

where P is the number of μg. of histamine diffusing across 1 cm.^{2} of the outer surface of the muscle, c_0 the initial concentration of diffusible histamine in μg./c.c., k the diffusion coefficient (cm.^{2}/min.) and t the time of diffusion in minutes. Since the various constants were not determined in each individual experiment the following average values have been assumed:

Initial concentration of diffusible histamine (c_0) 11·7 μg./g.

This value was obtained from Table 2 assuming 70% of the average histamine content of 16·7 μg./g. to be diffusible. This average may be too low owing to the

TABLE 2. Histamine content of rat's diaphragm before and after exposure to D-tubocurarine

		Histamine		
Conc. D-tubocurarine (mg./c.c.)	Duration exp. (min.)	Release (μg./g.)	Extract end of exp. (μg./g.)	Extract control (μg./g.)
2·0	20 + 10	11·6	8·0	—
Nil	0	—	—	15·9
Nil	20 + 10	—	—	24·0
2·0	20 + 10	7·0	8·7	—
Nil	0	—	—	24·2
2·0	30 + 90	11·2	4·7	—
Nil	120	—	—	14·5
8·0	30	10·5	9·1	—
Nil	30	—	—	17·0
Nil	60	—	—	14·0
4·0	30 + 30	—	3·9	—
2·0	30 + 30	—	5·8	—
1·0	30 + 30	—	7·5	—
Nil	—	—	—	19·3
Nil	—	—	—	18·3
1·0	90 + 90	13·5	4·9	—
Nill	0	—	—	14·8
1·0	90 + 90	9·3	2·6	—
Nil	0	—	—	11·0
1·0	90 + 90	11·9	4·4	—
Nil	0	—	—	15·6
1·0	90 + 90	—	4·1	—
Nil	0	—	—	11·6
			Means	
		10·7	5·8	16·7
		16·5		

inclusion of a rather atypical set of experiments with small rats (last group of Table 2). If these are omitted the average histamine content becomes 18·4 μg. and $c_0 = 12{\cdot}8$. This value would account better for the continuing linearity of regression line A in Fig. 5, since the highest values would still be less than 80% of the maximum.

Ratio of surface to weight: this was determined directly in a number of diaphragms, the average ratio being 27·2:1.

Ratio $P/\sqrt{t}$: this was obtained from regression line A (Fig. 5) after substituting corresponding surface for weight.

Substituting these values $K = 3{\cdot}9 \times 10^{-5}$ at 37°, or $3{\cdot}3 \times 10^{-5}$ using the larger value of c_0.

This may be compared with a diffusion coefficient of about 6×10^{-5} for urea for a diaphragm of the same thickness (Conway & Fitzgerald, 1942). Histamine thus seemingly diffuses through muscle about half as rapidly as urea.

The effect of temperature on the rate of release of histamine by curarine

The effect of three different temperatures on the rate of release is shown in Fig. 6. Each point is the mean of four observations. The results have again been plotted against the square root of time, in a manner appropriate to a diffusion process. The temperature coefficient for 10° of a diffusion process is of the order of 33%. The ratio of the slopes of two consecutive regression lines in Fig. 6 should therefore be approximately $\sqrt{1{\cdot}33} = 1{\cdot}15$. In fact, the ratio of slopes at 37 and at 27° is 1·19, but the ratio of 27 and 17° is 2·5. It is thus probable that within the range of temperatures of 27–37° diffusion is still the dominant factor and that the effect of temperature on diffusion can fully account for the slower histamine release at 27°. Below this range the effect of temperature appears to be much more complicated and may be due to an effect on the diffusion of curarine and/or the chemical process by which curarine releases histamine.

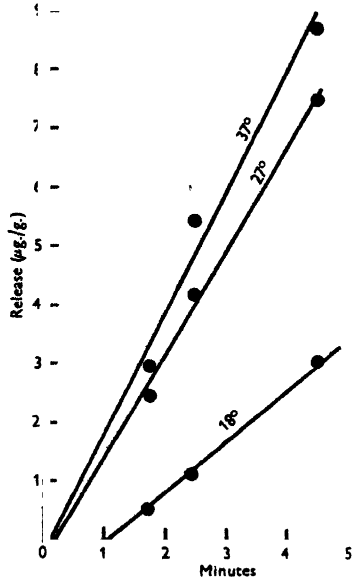

Fig. 6. Effect of temperature on rate of release of histamine (see text).

DISCUSSION

These experiments show that when rat's diaphragm is placed in a solution of curare approximately two-thirds of the histamine contained in it suddenly changes from an indiffusible into a diffusible state. It then diffuses out at a rate conforming to a simple diffusion process, the coefficient of diffusion being about half that of urea. The rest of the histamine which may be contained in some histologically distinct structure is not released by curare, although it can be released by other substances such as ammonia.

The effect of curare on histamine release depends critically on the concentration; if this is reduced to a quarter of the optimal, the rate of release becomes many times slower, and at a tenth of the optimal, it is only just perceptibly greater than in the controls. It will be shown in a subsequent paper that an extremely slow steady release of histamine occurs even in Tyrode solution alone, and it is possible that the effect of curare is greatly to accelerate a normally occurring process.

Little can be said at this stage about the mechanism by which curare produces this effect. Rocha e Silva (1943) has shown that histamine can form peptide linkages with certain amino-acids and has suggested that these might be chemical models of the way histamine is bound within the cell. It would be tempting to assume that curare and other bases which release histamine (diamidines, MacIntosh & Paton, 1947) did so by displacing histamine from its cellular 'receptors'. There is at present no positive evidence to support this assumption, but it can be shown that when histamine is released by curarine the amount of curarine involved would not only be sufficient to replace all the histamine on a molecular basis but usually exceeds it by a factor of 10. We have performed a series of experiments in which curarine was injected into the perfusate of rat's hindlimbs perfused with Tyrode solution. In this type of experiment, repeated injections of curare cause a repeated release of histamine, and very large totals may eventually be released. Table 3 summarizes some of these data and shows in each case the molar ratio of curarine injected to histamine released. The most favourable ratios were obtained when 0·3 c.c. of D-tubocurarine (1:100) were injected into the artery, but even then the ratio of molecules of curarine injected to molecules of histamine released was 20 or more. Relations of similar magnitude are found when instead of quantities, concentrations are compared. For instance, the concentration of bound histamine within the cell is of the order of N/5000, but the optimal concentration of

TABLE 3. Perfusion of rat's hindlimbs with Tyrode solution
Quantities of histamine released by single injections of D-tubocurarine

	D-Tubocurarine injected (mg.)	Histamine released (μg.)	Mol. curarine / Mol. histamine
R1 1st injection	3	12·4	31
2nd injection	3	16·8	23
R2	3	18·9	20
R3	2	5·0	51
R5	6	23·4	33
R7	3	16·4	23
R9 1st injection	6	35·6	22
2nd injection	6	31·1	25

curarine for histamine release is of the order of N/1000, a fivefold difference in favour of curarine. It may be concluded that chemical interaction between the two substances is feasible in view of the magnitudes involved, but that there is no positive evidence to suggest a stoichiometric relation between histamine released and curarine taken up. On the other hand, these results do not disprove a stoichiometric relationship, since the number of molecules of curarine taken up by the cell may be only a fraction of those injected.

As an alternative hypothesis, curarine may be producing 'tissue damage', or more concretely it may be activating certain enzymes which then release histamine. Gross histological damage at the neuromuscular junction following the injection of curare has in fact been described by Herrera (1936) and certain other less drastic but characteristic changes following the injection of curare have been described by Rojas, Szepsenwol & Resta (1939). On the other hand, McIntyre (1947) states that King & Willard were unable to find any significant change in the size of motor end-plates in rats after curarization with 1:1000 D-tubocurarine in Ringer solution. This may be a similar case to that of cholinesterase which was found to be inhibited by certain impure curare solutions but unaffected by pure D-tubocurarine (McIntyre & King, 1943). Dr R. E. Davies (Sheffield) has kindly tested for us the effect of D-tubocurarine on the oxygen consumption of the diaphragm using Warburg's technique. No decrease of tissue respiration was found in a solution of 1:1000 D-tubocurarine in Krebs Ringer. This excludes any gross interference with the respiratory functions of the cell. An interesting effect of curarine on potassium diffusion in frog's muscle was observed by McIntyre & King (1944) who found that tubocurarine (1:1000) approximately doubled the rate at which potassium diffuses from muscle.

SUMMARY

1. When the isolated diaphragm of a rat is placed in Tyrode solution containing 1:1000 D-tubocurarine at 37°, two-thirds of the histamine contained in the muscle diffuse rapidly into the surrounding fluid.

2. If the concentration of curarine is reduced the rate of release of histamine is greatly retarded, but if the concentration is increased the rate of release is not increased further.

3. A quantitative study of the rate of release suggests that, at the maximum rate, the liberation of histamine from diaphragm muscle can be treated as a simple diffusion process and reasons are given for assuming that the rate is determined by the rate of diffusion of histamine through muscle. On this assumption a 'diffusion coefficient' of histamine through muscle has been calculated. Its magnitude appears to be of the same order as that of urea.

4. The effect of temperature on the rate of release has been determined. The magnitude of the effect suggests that between 27 and 37° a simple diffusion process prevails.

5. Histamine is also released when rat's hindlimbs are perfused with Tyrode solution and curarine is injected into the perfusate. Under these conditions at least 20 molecules of D-tubocurarine are required to release 1 molecule of histamine.

6. Possible mechanisms by which curarine may cause a release of histamine are discussed.

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THE RESPIRATORY RATE AND VENTILATION IN THE NEWBORN BABY

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Proper treatment of respiratory insufficiency in very early life must obviously depend on a knowledge of the normal respiratory pattern in the neonatal stage. Dohrn (1895), using a spirometer with face mask and valves, made the first attempt to find data for normal respiratory behaviour in newly born babies. This instrument had a dead space of more than 50 c.c., which is now believed to be more than twice the average tidal air of the sleeping infant. Murphy & Thorpe (1931) used a body plethysmograph but the figures they obtained were very variable, probably due to restlessness on the part of the baby. It would seem to be necessary to design an instrument in which the infant will sleep as comfortably as in its own cot.

The difficulty with a plethysmograph is to effect a satisfactory seal, so that the mouth and nose are open to the outside air while the body is enclosed within the airtight instrument. Previous workers made the seal round the neck of the infant, generally using rubber sheeting coated with heavy grease. Any sealing device involving the neck must be applied most gently. Unfortunately, the circumference of the neck varies with crying, which causes vascular engorgement, and the contour of the neck changes with any movement of the head. It follows that confidence can only be placed in the adequacy of the seal if rigid tests are applied with monotonous frequency. Further, if there is any degree of congestion of the veins in the neck it will not be certain that there is no damming up of the drainage from the respiratory centre. Lastly, both from published results and from personal observation, it is known that it is very difficult to ensure that a normal infant sleeps peacefully while a neck seal is in place. Observations on the resting minute volume demand complete comfort and relaxation on the part of the subject.

THE PLETHYSMOGRAPH

The instrument (Cross, 1949) was constructed after some preliminary work on a plethysmograph based on the model of Deming & Washburn (1935). A reliable and comfortable seal is so important that a new approach was attempted copying a helmet used by divers in the Japanese navy in the recent war. This helmet was fastened to the head by means of a rubber seal which passed along the rami of the mandibles to the mastoid processes and so over the occiput. Almost the entire course of such a seal is over bony structures, so that a considerable pressure can be applied without impeding free flow along veins, arteries and trachea.

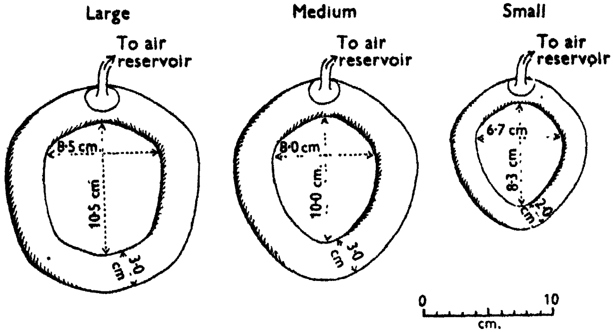

Fig. 1. Diagram to scale showing the three pneumatic cuffs.

By the kind co-operation of Mr Gorham of the Dunlop Rubber Co. a pneumatic cuff was constructed in the form of a shaped ring which was applied in such a way that it accurately framed the face of the baby when viewed from the front. The internal diameter of the ring fits across the sub-mental region of the jaw, crosses the mandible and passes along the ascending rami to the temporal region and so roughly along the coronal sutures to complete the circle near the anterior fontanelle. The outer circumference of the pneumatic cuff completes the seal by pressing against a wall built into the lid of the plethysmograph. The cuff is inflated from a reservoir, and a pressure between 45 and 60 mm. Hg is maintained within it to effect an airtight seal. Three cuffs have been constructed, for premature, for normal and for larger infants. Fig. 1 gives details of the shapes and dimensions of these cuffs. As the cuffs are made of very soft rubber and are inflated, it is not necessary to model an individual cuff for each baby. By adjusting the slope of the cuff within the walled enclosure of the lid it is possible to use the same lid for either the medium or the large cuff, a separate lid being used for the small cuff.

The plethysmograph is rectangular in shape, and is made of 14-gauge brass. The dimensions are 59 cm. long × 30 cm. broad × 26 cm. deep. The joints are made by bolting the sheets of brass to ⅜ in. angle brass, and by soldering to obtain an airtight junction. The upper edge of this brass box is finished with an out-turned flange of 1 in. angle brass. Particular care was taken to see that the up-facing flange formed a true plane. Vertical rods are soldered into the outer part of this flange to form guiding rails for the lid. This lid is made of ¼ in. Perspex, pierced by a hole near one end. This hole is 15·8 × 12·5 cm. and is lined with a wall of ⅛ in. Perspex, which projects 1 cm. above the level of the lid and approximately 4 cm. below it. When the instrument is in use the junction of the lid to the brass box is made airtight by laying a continuous ring of sponge-core rubber, ¼ in. in diameter, round the flange and internal to the vertical rods. The lid is held in position with twelve paper clips of the 'Bulldog' type (Fig. 2).

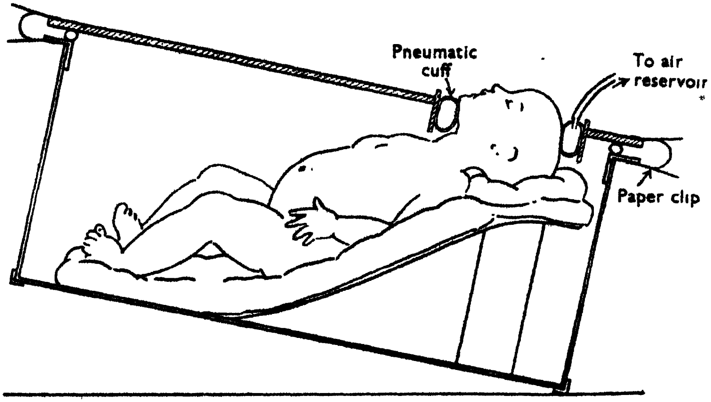

Fig. 2. Diagrammatic longitudinal section through the plethysmograph with a baby in position and the seals in place. In fact, the baby would be wearing its normal clothes and be covered with blankets.

Inside the plethysmograph there is a padded metal cradle for the baby. This cradle allows the infant to lie on its back, and supports the head so that the head lies naturally within the walled enclosure in the lid. The cradle slopes downwards from the head, to form an angle of about 25° with the floor of the plethysmograph. In order to prevent the baby from sliding down its cradle, the whole instrument is inclined at an angle of 10° towards the head. The result of these adjustments is that the infant's body slopes downwards at an angle of about 15° in the horizontal plane. This rather complex arrangement is necessary in order that the head may be slightly extended on the body, and thus keep the anterior chest wall of the infant well away from the lower lip of the walled enclosure of the lid. (It should be remembered that the newborn infant has a chest whose antero-posterior diameter is almost the same as its transverse diameter.)

The ink-writing volume recorder

If one can achieve comfort and a trustworthy airtight seal, the only problem remaining in measuring the volume of the infant's respiration is to have an accurate and sensitive volume recorder. This recorder has to be able to accommodate considerable volumes, so that a record of vigorous crying can be obtained. The seal must not be broken even by a maximal breath, and yet the recorder must produce a reasonable deflexion for such a small volume as 20 c.c. which is the average tidal air of a newborn baby.

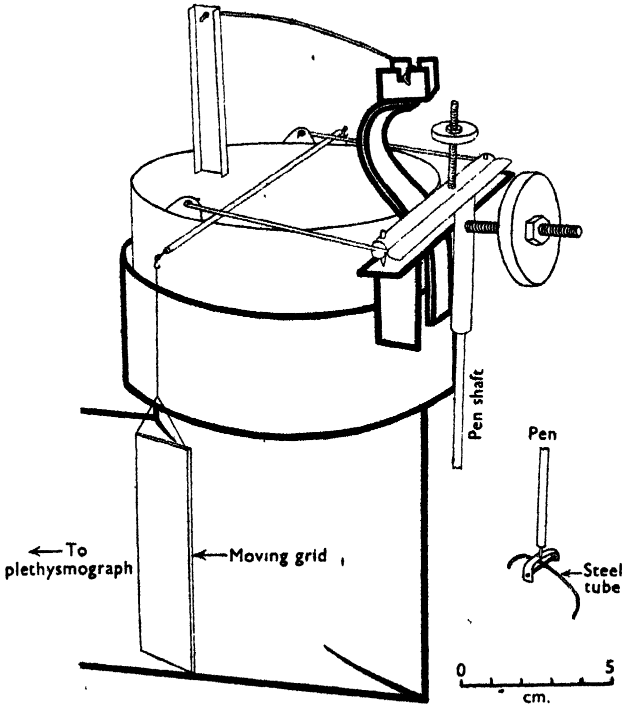

Fig. 3. Ink-writing volume recorder (semi-diagrammatic). All moving parts are drawn with thin lines, and stationary parts with thick.

The volume recorder is built into the upper part of one of the side walls of the plethysmograph, near the foot end, and the float chamber is adjusted to be horizontal when the instrument is at its normal 10° slope. In order to avoid the build-up of pressure and undue current formations, a short brass tube of 7·5 cm. diameter was used to join the volume recorder with the plethysmograph. The recorder itself is of the Krogh type. A kinematic suspension maintains the float horizontal, but permits vertical movement through a small arc of a large circle. The float is constructed of brass foil, six-thousandths of an inch in thickness, and all the attachments are made of the lightest possible material. In place of knife edges, which are difficult to adjust, the volume recorder pivots on two hardened steel points (gramophone needles). These points are correctly positioned when placed in two minute pits indented into a steel plate. The float is counter-balanced by two weights at right angles, which are adjustable on threaded brass (Fig. 3). It is possible to adjust the recorder so that it will maintain any position in which it is placed. As the sealing fluid, liquid paraffin, is somewhat viscous, and sticks to the wall of the float chamber, it was decided to adjust the counter-weights so that the volume recorder is slightly bottom heavy and comes to rest in the mid position. As it seemed desirable to have an instrument which would record faithfully the respiratory volume changes of an infant which might at times be breathing or crying at the rate of 80 or more respirations per minute, it was decided to sacrifice some of the initial high sensitivity by two methods of damping.

Damping was produced by the use of liquid paraffin, specific gravity about 0·8, as sealing fluid, and by making the float run up and down in an annular channel 0·7 cm. wide. Dynamic calibration showed that these two methods were effective in giving a faithful record of rapid changes in volume. In the early part of this work a frontal writing lever was used, with a smoked drum, but as the subjects for the study were newborn infants who may not be removed from their nursery, it was found to be much more convenient to use an ink-writing device. This was achieved by having a vertical lever which sweeps to and fro across a continuously passing stream of 'Manifoldia' paper. The pen consists of a No. 24 s.w.g. tube, which syphons the ink from a container set astride the paper.

Calibration of volume recorder

Two methods of calibration were used; static and dynamic. For the purpose of static calibration the plethysmograph was closed with a plain Perspex lid, and sealed in the usual way with paper clips. A test for leaks was performed by placing a weight of 9·5 g. (one penny) on the top of the float of the volume recorder. On removal of the penny the pen should return to its zero position. This test is the one routinely employed when the instrument is in use. When even a small leak was present, there was a rapid drift with the penny test. When the system was leak-proof, successive volumes of 20 c.c. of air were introduced into the plethysmograph through a brass tube soldered into the head end of the box. After a complete sweep of the paper, 20 c.c. volumes were successively withdrawn, and measuring and averaging of the results showed that there was no consistent variation between one side of the paper and the other, and the heights described did not vary among themselves by more than the error introduced by measuring them (0·5 mm.). By this static method of calibration it was found that a vertical deflection of 1 cm. represented a volume of 28·8 c.c.

Dynamic calibration

Though a baby's respiration is not represented by a simple harmonic wave, it was thought that the reliability of the volume recorder could be tested with a sine wave over a large range of speeds and volumes; an accurate response at a rate much higher than a normal infant's breathing, would justify confidence in results obtained from infants breathing at the lower rates. For this purpose a tube of 1·7 cm. diameter was soldered into the plethysmograph and was connected by a stiff rubber tube to an animal respiration pump from which all valves had been removed, so that a given volume of air was simply added to and removed from the plethysmograph by each stroke of the pump. The pump was set to deliver five volumes, between 7·2 and 102·2 c.c. per stroke (as measured by water displacement from a burette), and the speed was regulated to give ten rates between 11·3 cyc. and 144 cyc./min. The results of the dynamic calibrations for different volumes are given in the composite graph,

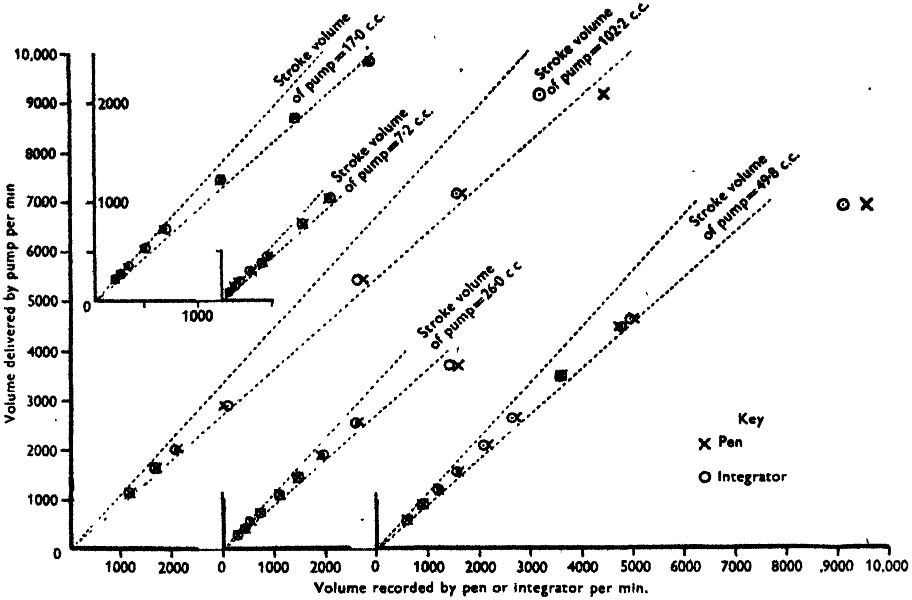

Fig. 4. Dynamic calibration of the volume recorder at five different stroke volumes of the pump. The scale for ordinate and abscissa is the same, but in order to keep the curves separate, the ordinate has been shifted for different curves. The dotted lines represent the 10% error lines for each curve, that to the left representing understatement by the pen or integrator, that to the right representing overstatement. For any given reading the number of cyc./min. of the pump may be calculated by

$$\frac{\text{volume delivered by pump/min.}}{\text{stroke volume of pump}}.$$

In the inset graph (top left) the scales have been doubled.

Fig. 4. It will be seen that there is an accuracy of ± 10% for the automatic integrator (see below) for any of the specified throws of the pump with rates up to 100/min.; but with the higher rates and larger volumes there is a considerable over-statement by the volume recorder. This is more pronounced by the pen than by the automatic integrator, the probable explanation being that the pen records not only the over-swing of the float, but also some 'whip' of its own.

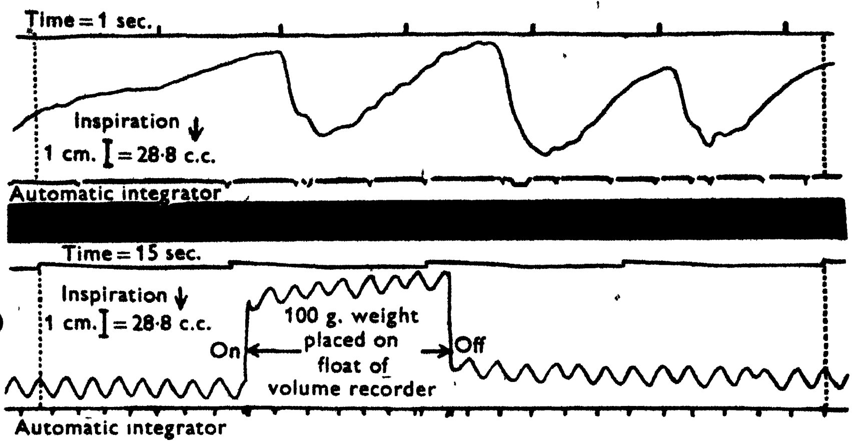

Fig. 5. (*a*) Tracing from baby crying vigorously. Note rapid inspiration. The volume recorder cannot record this rate of volume change faithfully. (*b*) Tracing from sleeping baby. The 100 g. weight on the float raised the pressure in the plethysmograph by 11 mm. of water. Note that there is no gross change in the volume or rhythm of the respiratory tracing. A leak is disclosed with this pressure.

This undoubtedly introduces a serious error with the crying infant where 100 c.c. or more may be inspired in 0·4 sec. (see Fig. 5*a*). Also, while crying the infant will move its head and open its mouth which will make the pneumatic cuff bulge farther into the plethysmograph and displace air. No attempt has been made to estimate the error involved in recording crying volumes. The results given here are from the sleeping infant only.

Error in reading results

In order to estimate the minute volume of the infant in c.c., one has to measure the vertical height in cm. of all the inspiratory movements recorded in 1 min., and multiply this height by the static calibration factor of 28·8. The thickness of the line described by the pen is less than 0·5 mm., and one aims to measure the heights to the nearest half mm.

Automatic integrator

The chief error in estimating the volume that the infant has breathed in a minute probably arises from fatigue on the part of the observer, for, in order to work out the results from an hour's trace, the height of many hundreds of lines must be measured. This operation is not only potentially inaccurate, but also very time-consuming, and, as it was hoped to undertake a considerable survey of newborn infants, Roberts & Widdas (1949) constructed an 'automatic integrator for volume recorders'. Fig. 4 shows a series of graphs of the overall dynamic calibration of the volume recorder, where the volume fed into the plethysmograph per minute is plotted against the volume described by the pen (from linear measurement) and by the automatic integrator. It is found that a deflexion of 1 cm. by the pen represents a volume change of 28·8 c.c. One count on the Post Office counter is equivalent to a volume change of 41·7 c.c.

As the pen describes a line which moves irregularly about a mean position on the paper, it is justifiable in using the automatic integrator to halve the volume recorded in order to measure the air inspired in 1 min. This is based on the assumption that the pen starts and ends each minute at its mean position. The errors involved from this assumption are greatly diminished if the volume of inspired air is averaged over a period of 5 min. or more.

By using this automatic integrator it is possible to obtain records from several babies in one day, and the results are immediately available. This compares very favourably with the 5 or 6 hr. work which had to be done on each tracing before the integrator was available.

'Back pressure' in the apparatus

Using the damping methods described above, it is apparent that the instrument may allow the build-up of a certain amount of back pressure. However, a water manometer attached to the plethysmograph gave a swing of less than 1 mm. with a sleeping infant. This may possibly be important since this apparently minute pressure may affect the infant's respiration and also may affect the accuracy in recording. To test, the first possibility, it was decided to increase the pressure within the plethysmograph greatly and to observe the infant for any signs of alteration in respiration. Weights of 50 and 100 g. were placed either on the float of the volume recorder or were suspended from the counter-weight. These weights gave pressures up to ± 11 mm. water. Examinations of the records taken under these conditions showed no change in respiratory rate and no gross change in volume (Fig. 5*b*). The actual volumes were not measured, as the volume recorder could not be trusted when so greatly out of balance. The second possibility seems to be ruled out by the overall dynamic calibration of the instrument.

TECHNIQUE

Selection of subjects. The subjects for this study were taken from normal babies delivered in the Maternity Wards of St Mary's Hospital.

No conscious method of selection was used. If the baby was judged to be normal by the clinician in charge, and the mother willing, the selection was generally made by the Sister in charge of the ward. At one point in the series it was found that many more females than males had been examined, and for a period the Sister was therefore asked to select a male child when possible. There was a certain prejudice in favour of getting repeated examinations on one child, in order to test the results of previous observers. It should be noted that in order to obtain the comparatively few results here published, over 100 infants have been placed in the plethysmograph, but only those who have fulfilled the criteria of rest defined below are quoted.

When the infant was first introduced into the plethysmograph, and after an airtight seal was obtained, it was found that there was a certain amount of baseline drift on the volume recorder, due to expansion of the air within the instrument as it was warmed by the infant's body. The small pipe which was soldered into the plethysmograph for the static calibration was connected to a three-way tap and well-oiled 50 c.c. syringe by means of rubber tubing, so that the volume of air in the plethysmograph could be conveniently adjusted without having to disturb the airtight seal.

When a record was to be taken, if the weather were cold, a warm rubber hot water bottle was placed in the instrument for a few minutes. The cradle was padded with two or three blankets and the infant was taken from its cot and placed in the plethysmograph. It was covered with the same number of blankets as were used in its cot, and the exact height of the padding beneath it was adjusted until it was thought that the head was suitably placed to fit centrally within the walled enclosure of the lid. The lid was now placed in position, and a finger passed between the lower lip of the wall and the infant's chest, to ensure that there was no contact here. If the position were satisfactory, a suitable cuff was placed round the head, and inflated from the air reservoir. After inflation, slight minor adjustments were made to remove creases or wrinkles. The paper clips were now applied to the lid, and the record commenced. A test for leaks was made with a penny, as described, and if this was satisfactory no further adjustments were needed.

Although the manipulations as described here may seem complicated, it was not at all unusual for an infant to be taken from its cot and placed in the plethysmograph, with an airtight seal, without waking the infant. More often, some crying occurred, but if the baby did not quickly settle down it was given a bottle of warm water to suck, and in nearly all cases this induced sleep. On a number of occasions the rectal temperature was taken before and after the hour's test. Except in the rare case of the fractious child, who cried vigorously throughout the examination, the rectal temperature was not increased.

To determine the minute volume of inspired air for the sleeping infant, it was necessary to define carefully a reasonable test period on a subject which is notoriously active. Figures given by other workers have been based on differing standards. Thus Deming & Hanner (1936) take 'representative half-minute periods', while Murphy & Thorpe (1931) select three half-minute periods from their traces, where the infant exhibited the slowest respiratory rates, and measured the volume inspired in this period. Only results obtained under the following conditions are presented:

(1) To minimize the metabolic stimulus of food, readings were taken only between 1½ and 3½ hours after meals. (The normal infants in this nursery are on a 4-hourly feeding regime.)

(2) The infant must have been quiescent for at least 20 min. before the part of the record used here commenced.

(3) The resting minute volume was obtained from a period of *not less than* 5 (up to 18) consecutive minutes, during which time the infant was sleeping quietly without any limb activity, facial grimaces or sucking movements. The minute volume is expressed as an average of such a period, provided that there were satisfactory tests showing no leak from the plethysomograph before and after the test period.

In practice the majority of the records lasted for about an hour, and in those where the infant rapidly settled down it was possible to show, by plotting minute volume against time, that the infant achieved a fairly steady resting level. The test was confined to this period of 1 hr. because of the potential danger from the pressure of the cuff round the face, which is near the systolic blood pressure of the normal infant, 80 mm. Hg (Woodbury, Robinow & Hamilton, 1938). In fact, no ischaemic changes occurred in the faces of the infants examined, beyond the expected temporary reactive hyperaemia.

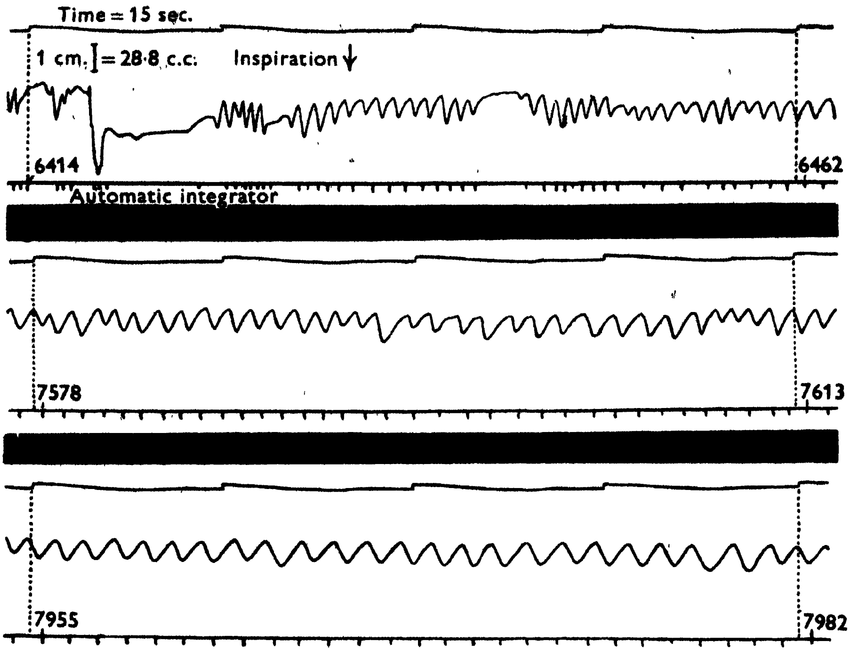

Fig. 6*a*.

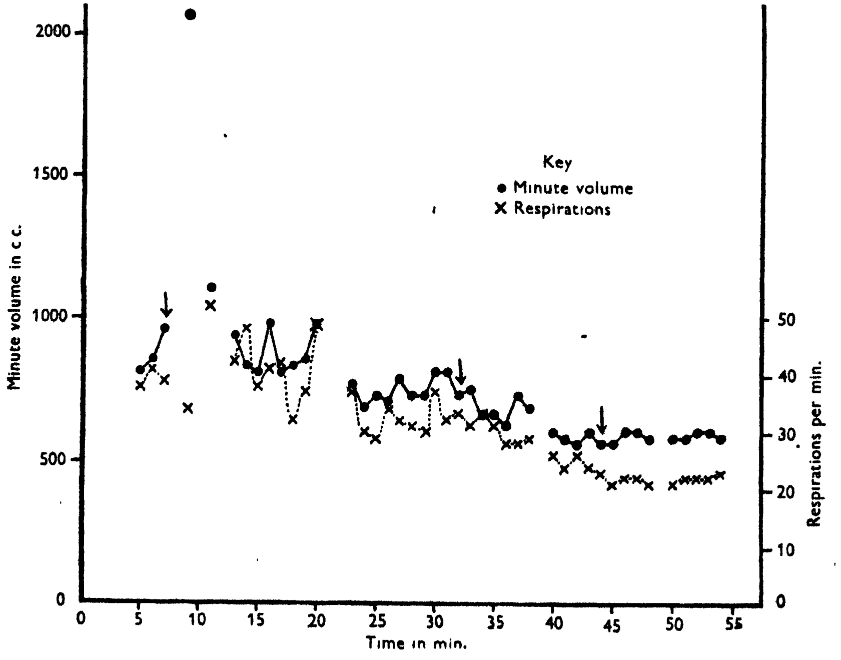

Fig. 6b.

For explanation of Fig. 6 see p. 469.

RESULTS

Three separate minutes of a trace from baby M1 are shown in Fig. 6, and with it the graph made for the whole period of the trace. This infant was chosen because the minute volume on this occasion approximates most closely to the mean minute volume for the whole series of babies examined.

TABLE 1

Date (1948)	Name	Sex	Age (days)	Weight lb.	Weight oz.	Period averaged (min.)	Minute volume Range (c.c.)	Minute volume Average (c.c.)	Respiratory frequency Range (per min.)	Respiratory frequency Average
17/3	D	M	4	7	2	5	511– 601	556	18–27	22·9
17/3	R	F	0·5	7	14	5	444– 598	542	23–30	26·0
22/3	H	F	1	8	6	5	504– 633	585	19–30	27·2
5/4	B	M	1	5	9	6	654– 739	690	28–38	32·4
22/10	M	F	0·5	8	7	7	591– 696	651	29–34	31·0
22/10	W	M	2	8	5	6	605– 688	661	34–39	36·5
26/10	W	M	6	8	10	7	646– 813	754	18–33	26·6
29/10	W	M	9	8	13	6	605– 729	677	20–29	25·3
22/10	W1	M	4	7	4	5	542– 625	600	30–35	32·5
25/10	B1	F	1	6	15	10	647– 730	683	34–41	36·8
25/10	G	M	3	6	12	12	605– 750	690	33–51	37·9
27/10	R1	F	13	7	3	8	584– 647	618	30–37	32·6
1/11	G1	M	3	10	15	12	917–1022	973	29–37	32·3
5/11	G1	M	7	11	0	12	708– 855	795	20–34	28·9
2/11	M1	F	3	7	2	11	438– 522	493	26–40	32·7
5/11	M1	F	6	7	0	9	479– 563	535	25–36	28·8
12/11	M1	F	13	7	7	9	563– 604	586	21–26	24·7
5/11	W2	F	10	8	4	12	667– 771	722	28–35	30·1
10/11	R2	F	5	7	5	6	583– 646	615	24–29	27·2
10/11	J	F	5	8	2	7	562– 646	622	19–22	20·8
10/11	M2	F	0·5	8	4	13	479– 646	557	25–33	28·3
13/11	M2	F	3	8	0	11	500– 646	567	33–44	37·8
17/11	M2	F	7	8	4	14	480– 563	526	25–32	28·4
19/11	C	F	0·2	6	14	12	313– 375	349	14–21	19·0
22/11	P	F	3	6	8	14	438– 585	513	28–36	33·2
26/11	P	F	7	6	12	14	543– 668	598	32–40	36·0
29/11	P	F	10	6	13	12	585– 688	624	29–36	33·3
29/11	J1	F	4	6	2	18	396– 647	493	18–35	24·7
30/11	W3	F	0·3	7	12	6	501– 585	539	20–25	22·7
1/12	W4	M	1	7	5	10	480– 668	543	23–25	24·0
3/12	H1	M	3	7	10	7	605– 668	638	38–41	39·6
8/12	L	M	4	8	2	7	354– 396	369	20–29	24·7
10/12	L	M	6	8	1	9	438– 485	466	22–33	29·1
13/12	S	M	9	6	13	9	480– 543	515	24–27	25·3
13/12	L1	M	4	6	12	6	417– 563	487	26–31	28·1
18/12	B2	M	7	7	4	7	354– 459	384	22–24	22·4

Table 1 gives the results from the infants who showed a satisfactory resting period as defined above. These are given in chronological order, save that repeated observations on one infant, made on different days, are brought

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Fig. 6 (*a*) Baby M 1, 12 November 48. Three separate minutes from the respiratory trace. (*b*) Graph of the whole trace from the same baby, with arrows marking the minute periods which are figured above. Where the graph is discontinuous, the tracing was not satisfactory for analysis because adjustments were being made, a leak was present or a test for leaks was being made. Between the 40th and 48th minutes the criteria mentioned in the text were satisfied, and so the average for this period has been accepted as the resting value.

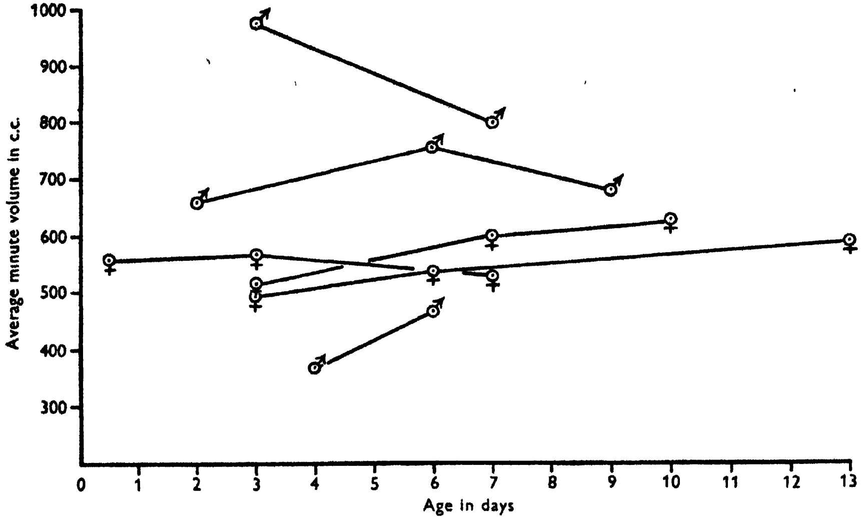

Fig. 7. Average minute volume in those babies who have been examined on more than one occasion. The straight lines link the repeated observations on one baby and are not meant to indicate any speculation on the volume a baby was breathing between observations.

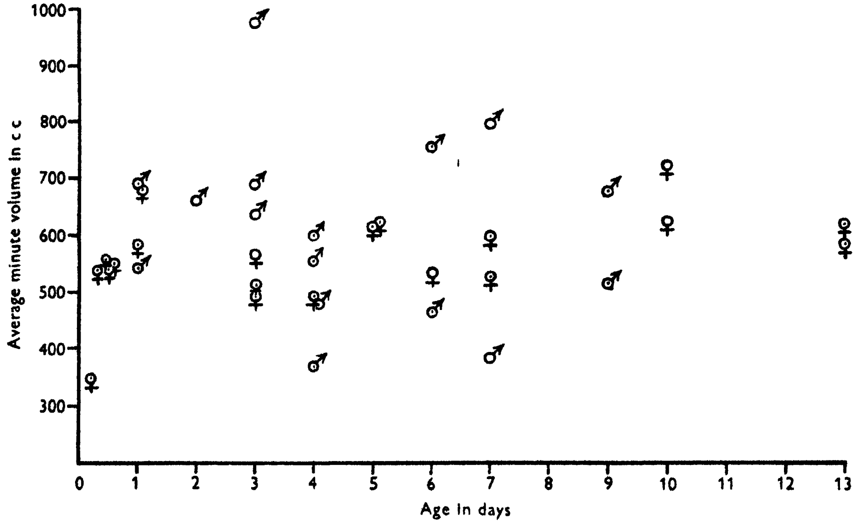

Fig. 8. Average minute volume in c.c. related to the age of the infant.

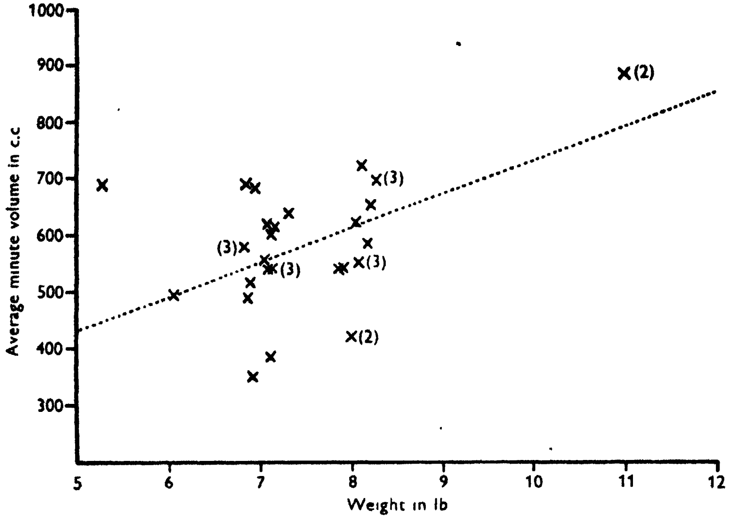

Fig. 9. Average minute volume in c.c. related to weight of subject. The numbers in brackets after six of the points indicate the number of separate records from this infant which have been averaged to give the weight and minute volume charted (see text). The dotted line is the calculated regression line based on these figures.

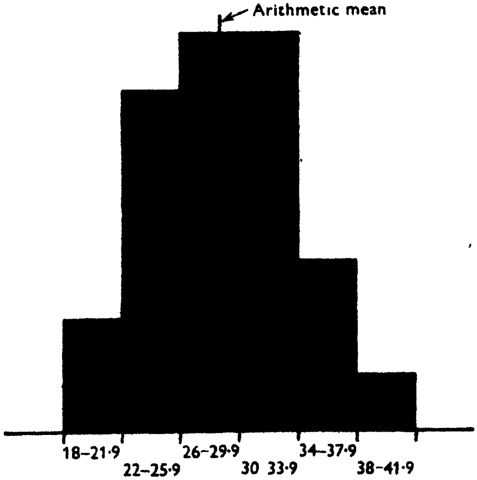

Fig. 10. Frequency distribution polygon showing average respiration frequency/min. in the sleeping infant. Where there are repeated observations on one infant the mean of the values has been taken.

together to facilitate comparison. It will be seen that in a particular baby during the first 13 days of life there is no consistent trend towards an increase of average minute volume with increase of age, which is contrary to the general belief, and to the statement of Murphy & Thorpe (1931) on rather inconclusive evidence from four babies. This point is illustrated in Fig. 7.

The average minute volume in c.c., plotted against the age and weight of the infants respectively, is shown in Figs. 8 and 9. In Fig. 9 the calculated regression line (see statistical note) is drawn. It was realized that it might well be of more significance to consider the minute volume of air breathed in relation to the surface area of the infant, but the only available information on surface area of infants was from the work of Lissauer (1903), in which he states that surface area is equal to $10{\cdot}3 \times \sqrt[3]{\text{weight}^2}$. This assumes that all babies are of identical shape and, in fact, for the weight range concerned, gives what is virtually a straight line relationship between weight and surface area. For this reason the weights of the infants, which are measurable, have been used rather than the hypothetical surface areas.

The frequency distribution polygon for the average respiratory rates of the infants studied is shown in Fig. 10. It will be seen that the shape suggests that a fair sample of infants has been examined. The average respiration rate is 28·64/min., with a standard deviation of ±5·195.

STATISTICAL ANALYSIS

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There are altogether thirty-six observations on twenty-six babies. Three observations are available for each of four babies, and two observations for each of two babies.

The data were examined in relation to two questions:

(*a*) Is there any evidence that, for an individual baby, the changes in A.M.V. (average minute volume) in the first 15 days or so of life are related to changes in weight or age?

(*b*) Disregarding the variation between successive readings on the same baby, is there any evidence that the variation between A.M.V. readings for different babies is related to the differences between the weights and ages of the babies?

The first question is answered by calculating the partial regression coefficients of A.M.V. on weight and age for those babies on whom more than one observation is made, each of the three variables being first reduced by subtracting the mean value for the baby concerned. It was found that neither of the partial regression coefficients of A.M.V. on weight or age (for fixed values of age or weight, respectively) were significant; nor were the ordinary regression coefficients of A.M.V. on weight or age. There is therefore no evidence in the present data that changes in A.M.V. for individual babies are related to changes in weights or age.

The second question is answered by calculating the partial regression coefficients of A.M.V. on weight and age, using the actual values for the different babies, and taking averages when there is more than one reading for any of the babies. The partial regression coefficient of A.M.V. on age for fixed weight is non-significant, but that of A.M.V. on weight for fixed age is significant. We may therefore ignore the effect of age and calculate the ordinary regression coefficient of A.M.V. (in c.c.) on weight (in oz.). This is found to be 3·6740 with a standard error of 1·1170. The value of Student's t is 3·289 (on 24 degrees of freedom), which would be exceeded by chance less than once in 100 times.

The mean A.M.V. is 589·33 c.c. with a S.E. of 19·35, and the mean weight is 122·528 oz., with a S.E. of 3·464. The equation of the estimated regression line is

$$\begin{aligned} y &= 589{\cdot}33 + 3{\cdot}6740\,(w - 122{\cdot}528) \\ &= 139{\cdot}17 + 3{\cdot}674w, \end{aligned}$$

y being the A.M.V. in c.c. and the w the weight in oz. The standard deviation of the scatter about the regression line is estimated as 116·1 c.c.

DISCUSSION

With this plethysmograph, in which it is possible to get the subject sleeping with some ease, it is to be expected that the average values obtained for respiration rate and minute volume will be lower than those of previous workers. In fact this is the case.

Thus Deming & Hanner (1936), in a study of eighteen normal infants in the first 11 days of life, find an average respiration rate of 44/min., with a range of 23–82/min. In the present series the average is 28·64/min., with a range between 14 and 51/min. Despite the satisfactory state of rest obtained, there are still quite striking variations when repeated 'satisfactory' examinations are made on one baby. Thus baby W shows rates of 36·5, 26·6 and 25·3 on different days. No explanation is offered for this, beyond the possibility that the criteria for rest are not sufficiently stringent.

Similarly, when dealing with average minute volumes, Deming & Hanner find an average which varies for different days of life between 734 and 1144 c.c./min. and a range between 225 and 1774 c.c. In this study the average is found to be 589 c.c./min., with a range between 313 and 1022 c.c./min. In view of the fact that these writers take their results from only ½ min. periods and it is possible for a baby to hold its breath for such a time, it is surprising that the limit of their range is not lower. One can only conclude that their subjects were far from experiencing normal rest.

Deming & Hanner found that there was a relationship between the average minute volume and the weight of the infant, and state that the volume of air breathed increases by something between 38·1 and 248·1 c.c./lb. body wt. The average value was 127·3 c.c., which is rather more than twice the value obtained in this study. Shaw & Hopkins (1931), in their study of premature infants, find a figure of 193 c.c./lb. body wt., which is equivalent to 10 c.c./oz. body wt. compared with the present figure of 3·674 c.c./oz.

SUMMARY

1. A new form of body plethysmograph for measuring the respiratory volume and rate of the newborn infant is described.

2. The seal is effected with a pneumatic cuff round the bony points of the face and head, thus leaving the trachea, veins and arteries free from obstruction.

3. With this instrument it is believed that one can approach normal resting conditions.

4. The mean average respiration rate of the infant in the first 13 days of life is 28·6 (S.D. = 5·2) resp./min.

5. The average minute volume of the infant in the first 13 days of life is found to be 589 c.c./min.

6. Evidence is given to show that there is positive relationship between the weight of the infant and the volume breathed per minute.

7. No such relationship is found for age.

My thanks are due to Prof. A. St G. Huggett, who suggested this problem to me, and who has given me generous help throughout. Also to Prof. A. D. M. Greenfield for a great deal of practical help in constructing the volume recorder, and to all the academic and technical staff of the Physiology Department. I am indebted to Dr Reginald Lightwood, who has allowed me free access to his patients and to Sister K. A. Taylor, of the Maternity Department and her staff for their helpful co-operation at all stages of the investigation. My thanks are also due to Dr Patrick Young, who clinically assessed the patients in the early part of this study and helped me in making some of the observations on them.

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THE EFFECT OF COCAINE ON THE ACETYLCHOLINE OUTPUT OF THE INTESTINAL WALL

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There is abundant evidence, obtained in various species of animals, to show that the different layers of the wall of the gastro-intestinal tract contain acetylcholine (Chang & Gaddum, 1933; Kahlson, 1934; Corteggiani, 1937; Shaw, 1938; Goffart, 1939; Hsu & Cheng, 1944; Welch & Hyde, 1944), which is synthesized there (Dikshit, 1938), and continuously released under physiological conditions and independent of the activity of the central nervous system or of the extrinsic nerves (Feldberg & Rosenfeld, 1933; Donomae, 1933; Donomae & Feldberg, 1934; Feldberg & Kwiatkowski, 1934; Gayet, Minz & Quivy, 1937; Goffart & Bacq, 1939; Feldberg & Solandt, 1942), but the problem as to which structural elements are the site of the acetylcholine metabolism is anything but settled. We have tried to approach this problem by examining how paralysis of the nerve cells in the intestinal wall by nicotine or by D-tubocurarine, or of the nerve cells and fibres, for instance by cocaine, affects the spontaneous release of acetylcholine.

In addition to extrinsic sensory and autonomic nerve fibres, the wall of the digestive tract contains two ganglionic nerve plexuses, the myenteric and the submucous. Their nerve cells are surrounded by a dense network of fine non-myelinated nerve fibres which disappear after section and degeneration of the vagi (Johnson, 1925) and which cannot be the site of the spontaneous release of acetylcholine because such section of the vagi diminishes neither the content nor the release of acetylcholine in the intestinal wall (Goffart, 1939; Goffart & Bacq, 1939). Practically every worker in this field has considered the myenteric and submucous plexuses as the sites of the acetylcholine metabolism; but there remains the possibility, so far only suggested by Hsu (1948), that the nervous elements in the intestinal wall are not the structures or not the sole structures responsible for acetylcholine synthesis. A non-nervous metabolism of acetylcholine certainly exists, as shown by the high content of acetylcholine and its

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synthesis in the nerve-free human placenta (Chang & Gaddum, 1933; Chang & Wong, 1933; Comline, 1946–7), and in the spleen of the ox and horse (Dale & Dudley, 1929; Comline, 1946–7). Thus the smooth muscle and gland cells of the wall of the digestive tract might themselves be the site of the acetylcholine metabolism. In that case, drugs with a selective action on nervous structures would not be expected to influence the spontaneous release of acetylcholine.

METHODS

The spontaneous release of acetylcholine was measured either directly by determining quantitatively the output of acetylcholine from a piece of intestine perfused with eserinized Locke's solution or indirectly, by taking the muscular contractions produced by eserine on the isolated intestinal preparation as evidence for the spontaneous release of acetylcholine. This we are justified in doing, because these contractions result solely from the inhibiting effect of eserine on cholinesterase, as the result of which the released acetylcholine then is no longer destroyed, accumulates and provokes its muscle-stimulating effects.

Effect of eserine on isolated intestinal preparations. The guinea-pig's and rabbit's ileum were used. When the contractions of the longitudinal muscle alone were recorded the preparation was suspended in a 16 or 20 c.c. bath of Tyrode solution. When in addition a record was taken of the intestinal volume, according to Trendelenburg's method, a 35 c.c. bath was used. For details see Feldberg & Lin (1949).

Output of acetylcholine from rabbit's perfused intestine. Rabbits were anaesthetized by intravenous injection of 4 c.c./kg. of a 25% urethane solution. The abdomen was opened and a cannula tied into the peripheral end of the superior mesenteric artery cut near the aorta. Perfusion was started with aerated warm Locke's solution. The details for removing and cleaning the intestine and its transfer to a perfusion chamber have been described elsewhere (Feldberg & Solandt, 1942). The perfused piece of intestine measured between 60 and 140 cm. but the values for the output of acetylcholine have been calculated for 100 cm. of intestine, which corresponds to approximately 20 g. tissue. After about 20 min. perfusion eserine in a concentration of 1 in 200,000 was added to the perfusion fluid. The perfusate was collected, starting 3–4 min. later, for 1 hr. The fluid oozing out from the intestinal lumen was collected, together with the venous effluent. When the effect of cocaine on the output of acetylcholine was examined the intestine was first perfused for 10–15 min. with Locke's solution containing cocaine, before the eserine was added and the collection of the perfusate was started. The samples were assayed for acetylcholine on the eserinized frog rectus muscle.

Synthesis of acetylcholine. Acetone-dried powders of brain and intestine were prepared, extracted with 0·85% NaCl and incubated for 1 hr. at 37° C. with adenosinetriphosphate, cysteine, choline, citrate, phosphate, $MgCl_2$, KCl, eserine and NaF according to the procedure described by Feldberg & Mann (1946). The samples were assayed for acetylcholine on the eserinized frog rectus muscle. Cocaine was used as hydrochloride, nicotine as acid tartrate, D-tubocurarine as chloride, eserine as sulphate, acetylcholine as chloride. The amounts given in the text always refer to the salt.

RESULTS

Responses of the intestinal preparation to eserine before and after nicotine, D-tubocurarine or cocaine.

When preparations of the guinea-pig's intestine were suspended in a 16 c.c. bath, the addition of 1–2 μg. eserine was often sufficient to produce a gradually developing contraction after a latency of a few seconds; 5 or 10 μg. produced this effect regularly. At the end of 60–90 sec. the eserine was washed out and after some delay the muscles gradually relaxed. The eserine effect could be obtained repeatedly on the same preparation without great quantitative variations.

It is known that the stimulating effect of eserine on the intestine is not abolished by nicotine in doses which paralyse the nerve cells in the intestinal wall (Heathcote, 1932). We confirmed this, using very small doses of eserine. In addition we found that D-tubocurarine, which also paralyses ganglion cells in the intestine (Feldberg & Lin, 1949), did not prevent the eserine contractions. The paralysis of the ganglion cells was shown by the loss of the stimulating action of small doses of nicotine.

The results obtained with cocaine were similar to those obtained with nicotine or D-tubocurarine. Care had to be taken not to use cocaine in too high concentrations, which would paralyse the muscle fibres as well and thus render the preparation not only insensitive to eserine but also to acetylcholine or histamine. It is possible, however, with cocaine concentrations of 1 in 80,000 or less, to paralyse the nervous structures without materially affecting the excitability of the muscle fibres (Feldberg & Lin, 1949). In this condition nicotine no longer produces its stimulating effect, but eserine still causes contraction. In some experiments in which somewhat stronger concentrations of cocaine were used the response to eserine was affected to a somewhat greater extent than that to acetylcholine, but this quantitative difference could not be obtained regularly, so that no great significance could be attached to it.

There are certain differences in the response to eserine after paralysis of the nervous structures in the intestinal wall. These become evident when the changes in intestinal volume are recorded. For these experiments the rabbit's ileum was used, the muscle fibres of which are less sensitive to cocaine than those of the guinea-pig's ileum. With a slight negative pressure in the lumen of the intestine its walls are in apposition and the volume record is a horizontal line. In this condition eserine causes strong contraction of the longitudinal muscle. When the muscle has become more or less maximally contracted, contractions of the circular muscle layer set in; these can be seen with the naked eye and result in lengthening the preparation as shown in Fig. 1A. When the nervous elements are paralysed by nicotine, D-tubocurarine or cocaine, the only change in the action of eserine is that the contractions of the circular muscle occur less frequently and are less pronounced.

With a positive pressure in the intestinal lumen the preparation is greatly shortened and exhibits peristaltic contractions. In this condition or when the peristaltic contractions have come to an end, due to fatigue, eserine causes some further shortening, but its main effect is a strong tonic contraction of the circular muscle, which begins with slight rhythmic contractions at a rate similar to that of the rhythmic contractions of the longitudinal muscle (Fig. 1B). The tonic contraction of the circular muscle does not completely obliterate the lumen of the intestine; usually the upper (stomachal) end is slightly more contracted, so that the preparation assumes the shape of a cone. Fig. 1A, B illustrates the difference in the responses of the two muscle layers to eserine with negative and positive pressure in the lumen.

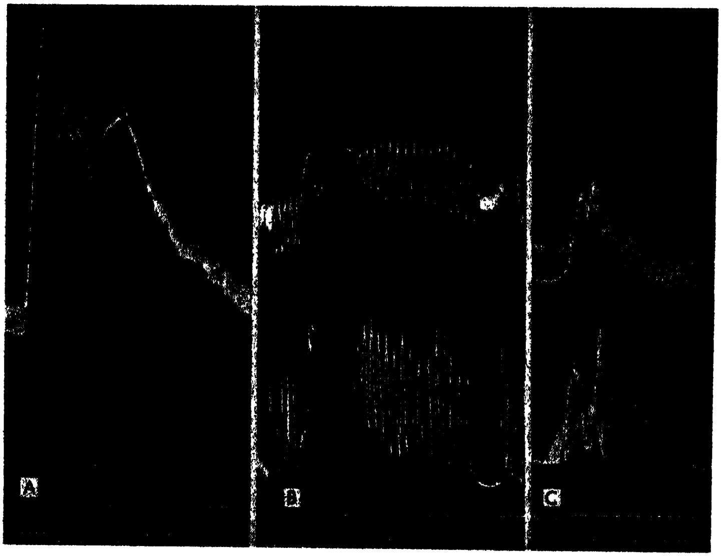

Fig. 1. Rabbit's ileum in 35 c.c. bath. A, B and C 20 μg. of eserine for 2½ min., C in the presence of cocaine hydrochloride 1 in 33,000. Upper record: contraction of longitudinal muscle. Lower record: intestinal volume; at A slight negative, at B and C positive pressure of 3 cm. saline in intestinal lumen. Time in 30 sec.

After nicotine, D-tubocurarine or cocaine a positive pressure in the intestinal lumen no longer initiates peristalsis. Eserine then produces strong rhythmic contractions and increase in tone of the circular muscle (Fig. 1C). In some preparations the effect on tone, in others that on the rhythmic contractions was more pronounced. The rhythmic contractions were easily distinguishable from the peristaltic waves since they did not spread from the stomachal end but affected the whole muscle simultaneously, so that the lumen of the intestine became alternately obliterated and filled with saline solution. In addition, the contractions were now more frequent than the peristaltic waves and occurred synchronously with the rhythmic contractions of the longitudinal muscle, which also showed some increase in tone. Thus paralysing the nervous structures in the intestinal wall abolishes the myenteric reflex but not the response to eserine which then consists of rhythmic and tonic contractions in both muscle layers.

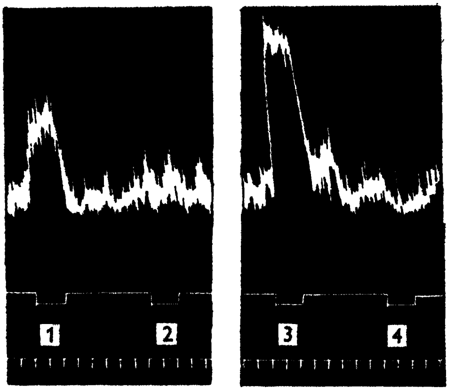

Fig. 2. Guinea-pig's ileum in 20 c.c. bath. 1 and 2, 0·4 mg., 3 and 4, 1 mg. cocaine hydrochloride for 1 min. The cocaine solution at 1 and 3 had been boiled in N/60 NaOH solution for 1 min. and neutralized before use. Time in 30 sec.

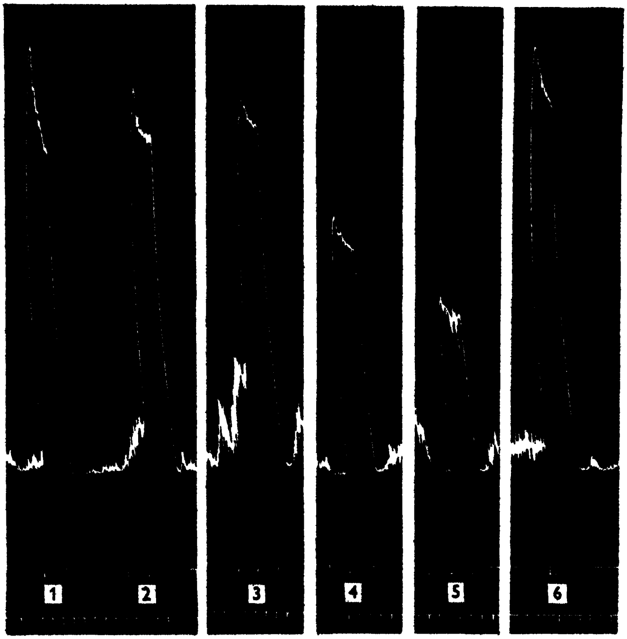

Fig. 3. Effect of 0·1 μg. acetylcholine for 1 min. on guinea-pig's ileum in 20 c.c. bath. At 2 and 4, 1 mg., at 3 and 5, 2 mg. cocaine hydrochloride added to bath 1 min. before the acetylcholine, and washed out with it. The cocaine at 2 and 3 had been boiled in alkaline solution and neutralized. Time in 30 sec.

Stimulating action of cocaine

We have previously shown (1949) that cocaine occasionally causes well-maintained contractions of the guinea-pig's ileum preparation, but we were unable to specify conditions which might bring about this effect. We have now found that solutions of cocaine acquire this stimulating property regularly when they are boiled in about N/60 NaOH for a minute or two and then neutralized (Fig. 2). Cocaine in sufficiently high concentration decreases the excitability of the muscle fibres to drugs like acetylcholine or histamine. This effect is greatly reduced when the solution is boiled in alkaline solution for a short time and has acquired its stimulating property. In the experiment of Fig. 3 1 mg. of untreated cocaine inhibited the acetylcholine contraction more than 2 mg. cocaine boiled in alkaline solution. The figure also shows that the cocaine had acquired its stimulating property.

When cocaine was boiled in acid it occasionally produced a very slight contraction of the ileum which was trivial compared with the contraction produced after boiling with alkali. After boiling at neutral pH or standing in alkali at room temperature cocaine did not acquire the stimulating property.

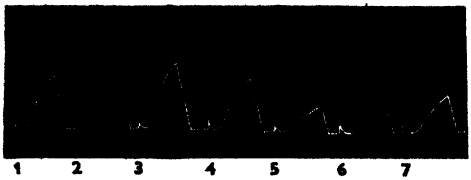

Fig. 4. Contractions of eserinized frog's rectus muscle to acetylcholine 1 in 25 millions, each time for 90 sec.; 4½ min. intervals. At 3 and 5 with cocaine hydrochloride 1 in 25,000, but at 3 the solution of cocaine had been boiled in alkaline solution and neutralized.

Examination of the effect of alkali-treated cocaine solutions on the intestinal preparation was suggested by observations made when assaying tissue extracts for acetylcholine on the frog's rectus muscle. For such assays the extracts are tested against acetylcholine solutions to which extract in an amount equivalent to that used for preparing the test solution is added, after the acetylcholine of the extract has been destroyed by brief boiling in alkaline solution. This procedure was found not to be applicable to extracts which contained cocaine because the cocaine present in the extract depressed (Fig. 4), but after boiling in alkaline solution augmented, the acetylcholine response of the frog rectus muscle. In some instances this augmenting effect of boiled cocaine solution was seen in a concentration of as little as 1 in 125,000.

Effect of cocaine on the synthesis of acetylcholine

The acetylcholine-synthesizing property of acetone-dried tissue of the intestinal wall has not been described hitherto. Details of results obtained in different species and with the different layers of the wall of the intestine will be described elsewhere.

When assaying the final extracts after incubation provision had to be made for the fact that cocaine depresses, and after boiling at an alkaline pH, augments the acetylcholine contraction of the frog rectus muscle, as stated above. To avoid errors arising from this source one of the following two procedures was adopted for the assay: (1) Two similarly prepared samples containing the same concentration of cocaine were incubated; one was used as a blank and contained inactivated enzyme. Inactivation was brought about by brief boiling of the saline extract prepared from the acetone-dried tissue (brain or intestine). After incubation the active sample was assayed against acetylcholine solutions to which equivalent amounts of this blank sample had been added. (2) The usual procedure of dividing the incubated sample into two parts and destroying the acetylcholine in one by boiling it at an alkaline pH was retained, but before testing, fresh cocaine was added to this part and an equal amount of a cocaine solution, which had been boiled in alkali, to the other. The amounts of cocaine used were such that both the test solution and the control extract contained finally the same amount of untreated and alkali-treated cocaine.

With these precautions the acetylcholine values obtained for the synthesis of acetylcholine were the same whether the synthesis had taken place in the absence or presence of cocaine (Table 1). We may mention in this connexion that chloralose and sodium bromide, in concentrations up to 1 in 500, do not affect the synthesis of acetylcholine in saline extracts prepared from acetone-dried brain.

TABLE 1. Effect of cocaine on synthesis of acetylcholine in saline extracts of acetone-dried brain (Exps. 1–3) and intestine (Exps. 4 and 5)

	Acetylcholine synthesized in 1 hr. (μg./g. acetone powder)		
Conc. of cocaine ...	0	1:10,000	1:1000
Exp. 1	670	670	710
Exp. 2	750	750	750
Exp. 3	930	—	800
Exp. 4	100	105	105
Exp. 5	170	190	200

Effect of cocaine on the acetylcholine output from the perfused intestine

Cocaine, even in high concentrations, does not interfere with the output of acetylcholine from the perfused small intestine of the rabbit (Table 2). The acetylcholine values for the control experiments were partly taken from the paper of Feldberg & Solandt (1942); their perfusions were performed under exactly similar conditions. In testing the samples for acetylcholine no difficulties were encountered from the presence of the cocaine, probably because it had been largely destroyed in the course of the perfusion.

TABLE 2. Effect of cocaine on the output of acetylcholine from the small intestine of the rabbit perfused with eserine 1 in 200,000

Conc. of cocaine in perfusion fluid	Output of acetylcholine during 1 hr. perfusion (μg./100 cm. intestine)
None	1·5, 1·8, 2·4, 3·5, 4·5, 4·2, 5·5
1 in 50,000	5·8
1 in 25,00	1·4, 5·8
1 in 10,000	2·0, 6·7
1 in 800	3·3, 6·1

With the exception of the experiments in which cocaine 1 in 800 was present in the perfusion fluid, the released acetylcholine also affected the behaviour of the perfused intestine. After a latent period of about a minute after the addition of eserine to the perfusion fluid the longitudinal muscle started rhythmic contractions which led to swaying movements of loops of the intestine; strong contractions of the circular muscle followed and, at stages, the main effect consisted in tonic contraction of this muscle layer in most parts of the perfused intestine. These muscular effects after eserine were absent when the perfusion fluid contained cocaine 1 in 800, and the muscle fibres had become insensitive to the released acetycholine. On the other hand, the high concentration of cocaine apparently did not inhibit the activity of the gland cells, because the effluent from the lumen was not a clear fluid but seemed to contain a profuse secretion as shown by its very cloudy appearance.

DISCUSSION

Our experiments have shown that neither paralysis of the nerve cells by nicotine or D-tubocurarine nor of the nerve fibres by cocaine inhibits the spontaneous release of acetylcholine in the wall of the small intestine. It was already known that eserine exerts its stimulating effect on the intestine during nicotine paralysis, and Feldberg & Rosenfeld (1933) had found that acetylcholine continued to be released from the perfused intestine when paralysing concentrations of nicotine had been added to the perfusion fluid. Thus there was previous evidence to show that, in the intestinal wall, activity of the nerve cells and release of acetylcholine are independent phenomena. Nevertheless, the nerve cells remained the structures to which the acetylcholine metabolism was most commonly attributed. We have confirmed that acetylcholine release in the intestinal wall is not the result of ganglionic activity. Our experiments with cocaine go even further. They suggest that the release is also independent of the functioning of the nerve fibres. This is in striking contrast to the effect which cocaine exerts on the release of acetylcholine from the endings of preganglionic sympathetic nerve fibres. In experiments on the perfused superior cervical ganglion of the cat, Harvey (1939) found that procaine not only rendered the ganglion cells insensitive to acetylcholine but also prevented its liberation from the preganglionic endings during stimulation of the cervical sympathetic. Recently Emmelin, Feldberg & MacIntosh, in unpublished experiments, have confirmed this result for cocaine. In a concentration of 1 in 20,000 cocaine practically prevented the output of acetylcholine from the ganglion during stimulation of the cervical sympathetic.

At the present stage of our knowledge it is not possible to give a definite and satisfactory explanation for this difference, but the following possibilities have to be considered: (*a*) the nerve fibres of the myenteric and submucous plexus may be more resistant to cocaine than those of the cervical sympathetic;

(*b*) excitation of the nerve fibres of these plexuses may not be necessary for the release of acetylcholine; (*c*) the acetylcholine metabolism of the intestinal wall may not originate in its nervous structures.

(*a*) Perhaps we are not justified in comparing the sympathetic nerve fibres with those of the plexuses in the intestinal wall. The plexuses have often been looked upon as primitive central nervous systems and so we may regard their fibres as having the properties of fibres of a central nervous system. We do not know, for instance, if cocaine would stop the spontaneous release of acetylcholine from incubated tissue slices of brain or if it would leave this release as unaffected as that from the intestinal wall. On the other hand, cocaine abolishes the peristaltic reflex, and to explain this by an action on the nerve cells and not on the nerve fibres would be to postulate a difference in the sensitivity of the two nervous structures to cocaine for which there is no analogue elsewhere. In addition, the concentration of cocaine used in some of the perfusion experiments was great enough to affect the muscle fibres directly. Thus we should have to make the unconvincing assumption that the non-myelinated nerve fibres of the myenteric and submucous plexus are more resistant to cocaine than the muscle fibres themselves.

(*b*) There is no inherent difficulty in assuming that some kind of ionic instability exists in the nervous elements of the plexuses by which a continuous release of acetylcholine is effected without excitation of the fibres. In that case the release would not necessarily be abolished by cocaine which blocks nervous conduction. However, experimental evidence for such an assumption is lacking.

(*c*) The idea that the nervous structures in the intestinal wall are not responsible for the acetylcholine metabolism certainly provides a simple and sufficient explanation as to why cocaine does not prevent the release of acetylcholine, but such a far-reaching conclusion can hardly be justified by this fact alone. Previous attempts to determine whether the plexuses were responsible for the acetylcholine metabolism have given conflicting and indecisive results, although the main evidence indicated these structures. For instance, Dikshit (1938) stressed the role of the ganglion cells because he had found that synthesis of acetylcholine in the *muscularis externa* was a property practically confined to the longitudinal muscle layer to which the main bulk of the ganglion cells adhere when it is stripped off the circular muscle layer; that pieces of intestine lost their ability to synthesize acetylcholine when kept in the refrigerator for a few days, which entails degeneration of the nerve cells; and that in the embryo the intestinal wall did not acquire the ability to synthesize acetylcholine before the plexuses had developed. Of these three findings the second could be accounted for by the fact that the enzyme responsible for the synthesis of acetylcholine becomes inactive when tissue or tissue extracts are stored in the cold (Mann, Tennenbaum & Quastel, 1938; Nachmansohn & Machado, 1943; Feldberg, 1944). Furthermore, Goffart (1939) found that the variations in the acetylcholine content of the whole wall of the *muscularis externa* in the different parts of the digestive tract of the dog bore no relationship to the density of the ganglion cells of the myenteric plexus. On the other hand, Hsu & Cheng (1942) claim to have obtained such a parallelism in the rabbit. Unfortunately their values for the acetylcholine content are many times higher than those obtained by any other worker, and the individual variations are so great as to render the comparison practically valueless.

Further experiments which have to be considered in this connexion are those by Welch & Hyde (1944), who, by mapping out the areas and the thickness taken up by the nerve cells of the myenteric plexus, estimated that in the rabbit they constitute about 2·5 % of the mass of the tissue of the stripped-off longitudinal muscle layer. Its acetycholine content/g. tissue was three to four times higher (i.e. 10–16 μg./g.) than that of the whole wall. If all the acetylcholine were derived from the ganglion cells, these would contain 400–640 μg./g. acetylcholine. The stripped-off longitudinal muscle of the guinea-pig's ileum also yielded on extraction an acetylcholine equivalent (16–20 μg./g.) several times higher than the whole wall, although in this animal the ganglion cells are said to adhere to the circular muscle layer of the intestine (Irwin, 1931). Welch & Hyde were not aware of this finding, but they assumed the same distribution of the ganglion cells as they had found in the rabbit, and considered the possibility of an even higher acetylcholine content of the myenteric ganglion cells in the guinea-pig than in the rabbit.

Hsu (1948) has pointed out that such high values for an acetylcholine content of nervous tissues are encountered nowhere else, and the calculations leading to these figures therefore may be based on the erroneous assumption that the acetylcholine of the intestinal wall is derived solely from the ganglion cells. He stresses the fact that sympathetic ganglia in the cat contain at most about 40 μg./g. acetylcholine, but he overlooks that it is present in a small fraction of the total mass of ganglionic tissue only, i.e. in the preganglionic nerve endings which therefore must contain the acetylcholine in a much higher concentration. In addition, the values given by Welch & Hyde for the acetylcholine content of the longitudinal muscle layer are probably too high because: (*a*) in their method of extraction the tissue was ground in eserinized saline solution before acidification, so that synthesis of acetylcholine must have taken place; and (*b*) no allowance was made when assaying the extracts for their high choline content, The difference between the acetylcholine content of sympathetic ganglia and longitudinal muscle layer, therefore, is less striking than it appears at first sight.

Instead of trying to answer the question of the origin of the acetylcholine in the wall of the digestive tract from inconclusive evidence it seems more profitable to emphasize that the origin is not settled and that the results obtained with cocaine in particular make it necessary to repeat and extend some of the previous experiments. In devising such experiments it will be well to keep in mind that, even if the nervous structures are responsible for the acetylcholine metabolism, this does not necessarily mean the nerve cells. In the peripheral and probably also in the central nervous system, acetylcholine is present in the axon, but there is no evidence for its presence in the cell body to any great extent. Thus comparisons in which the acetylcholine metabolism is related to the number of nerve cells have to be treated with a certain reserve. When trying to associate the acetylcholine metabolism with certain structural elements in the intestinal wall we must also remember that the wall is the site of a tremendous choline turnover. Large amounts of free choline are present in the intestinal wall (Kinoshita, 1910; Kahlson, 1934; Shaw, 1938) and are continuously released from it, as shown by Weiland (1912) and Le Heux (1918/19). But this release does not diminish the free choline content of the tissue; choline is apparently split off continuously from the large amounts of lecithin present in the intestinal wall (Abderhalden & Paffrath, 1926). According to Kahlson (1933) 1 g. guinea-pig's intestine contains about 1·5 mg. of choline bound as lecithin. How far the choline metabolism is associated with the nervous plexuses in the wall of the digestive tract is still an open question, and so is the interesting one of its association with the acetylcholine metabolism.

The incidental observation of the stimulating action of alkali-treated cocaine has not been followed up. It is possible that the treatment of the cocaine solution with alkali and heat resulted in the splitting off of ecgonine and that this base exerts the stimulating effect. In that case an enzymatic action of this kind could be visualized to occur in the living tissue and to be responsible for at least some of the stimulating actions of cocaine. Since our previous communication we have come across a paper by Salant & Parkins (1932) who observed stimulating effects of cocaine on the intestinal preparation where the pH of the bath fluid was either on the alkaline or acid side. The stimulating effect observed in the former condition may have been due, as in our experiments, to an action of the alkali on the cocaine molecule and not to a change in excitability of the intestinal muscle as assumed by these authors.

SUMMARY

1. The spontaneous release of acetylcholine from the wall of the small intestine of the guinea-pig and rabbit has been studied under conditions in which the nervous structures are paralysed. The eserine-produced contractions of the intestinal preparation suspended in a bath of Tyrode solution were taken as evidence for this release. In addition, the acetylcholine was assayed in the perfusate from intestines perfused with eserinized Locke's solution.

2. Paralysis of the nerve cells in the intestinal wall by nicotine or D-tubocurarine, or of the nerve fibres by cocaine does not prevent the stimulating action of eserine on the isolated preparation. Eserine then produced strong rhythmic contractions and an increase in tone in both muscle layers. The effect

on the circular muscle is seen when a volume record is taken from the intestine with a positive pressure in the lumen.

3. The spontaneous output of acetylcholine from the perfused rabbit's intestine is not abolished or reduced when cocaine hydrochloride even in a concentration of 1 in 800 is present in the perfusion fluid. This strong concentration paralysed the nervous structures as well as the muscle fibres.

4. Cocaine does not inhibit the synthesis of acetylcholine in saline extracts prepared from acetone-dried tissue of brain or intestine.

5. The fact that paralysis neither of the ganglion cells nor of the nerve fibres in the intestinal wall prevents the release of acetylcholine is discussed. The nervous structures in the intestinal wall may be the site of the acetylcholine metabolism: in that case the spontaneous release would appear to be independent of excitation of the nerve fibres of the plexuses and thus be different from the usual release of acetylcholine at cholinergic nerve endings. Alternatively, the acetylcholine metabolism in the intestinal wall does not originate in its nervous structures.

6. Solutions of cocaine boiled for a short time at an alkaline pH acquire a stimulating effect when tested on the intestinal preparation and augment the acetylcholine response of the frog's rectus muscle.

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THE RICKETS-PRODUCING AND ANTI-CALCIFYING ACTION OF PHYTATE

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It was shown that in the absence or deficiency of a fat-soluble calcifying vitamin, cereals had a rickets-producing effect on puppies, for the greater the amount eaten the more intense the resulting disease (Mellanby, 1919, 1920, 1921). Later it was found that there was also a qualitative difference between cereals; for, although the rates of growth of the animals were approximately equal, more severe rickets developed when the diet consisted mainly of oatmeal, maize or whole wheat flour than when these substances were replaced by equal amounts of either white flour or rice, in spite of the fact that the former cereals contained more calcium and phosphorus than the latter (Mellanby, 1922, 1925). The problem was therefore not merely one of growth promotion, but also of the presence in the different cereals of varying amounts of an anti-calcifying substance which interfered in a positive way with calcium metabolism.

This evidence was strengthened by a further observation that the rickets-producing effect of oats could be reduced either by boiling them with a mineral acid or by subjecting them to a malting process (Mellanby, 1925). Later analysis by M. Mellanby (1929) of the effect of treated cereals on the calcification of teeth showed that germination of the grain, which is the first stage of the malting process, did not in itself greatly reduce the anti-calcifying effect, but that if, after germination, the grain was minced and allowed to autolyse, the teeth were less defective in structure. Thus it became more and more certain that cereals contained a chemical agent which interfered with calcium metabolism and that their anti-calcifying effect could be reduced by chemical, and possibly enzymic, action.

In the light of the recent widespread attention that the subject of anti-vitamins has received, it is of interest to note that the term was first used in the course of this work on cereals in relation to calcification, when it was stated—'As regards the mode of action of this substance, its influence is so drastically opposed to that of the anti-rachitic vitamin that the possibility of its being an anti-vitamin suggests itself' (Mellanby, 1925). In 1926 the word anti-vitamin was replaced by the word toxamin—a substance having a toxic action which could be antagonized by a vitamin (Mellanby). In the present paper, after the experimental results have been discussed, this problem will be considered in relation to the general subject of biochemical antagonism, which has become so important in recent years.

The work was carried a stage further when Harrison & Mellanby (1939) showed that one substance in oatmeal which conferred upon it a powerful anti-calcifying action was phytic acid. Thus it appeared probable that, when oatmeal or oats were boiled with dilute HCl, the cereal phytate was hydrolysed to inositol and phosphate, and when oats and other cereals were malted, a similar change was effected by a phytase present or produced in the germinating grain; also that the reduction in the anti-calcifying action of the treated products was due to this hydrolysis of the phytate.

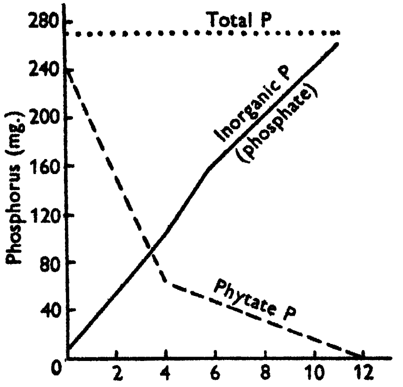


Fig. 1.

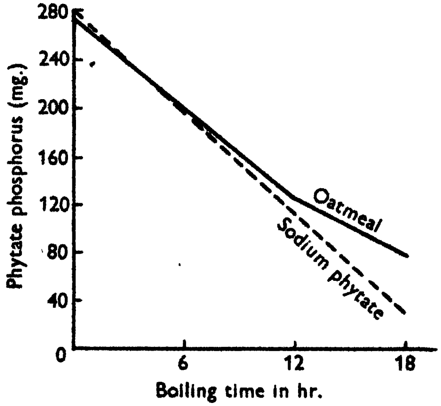


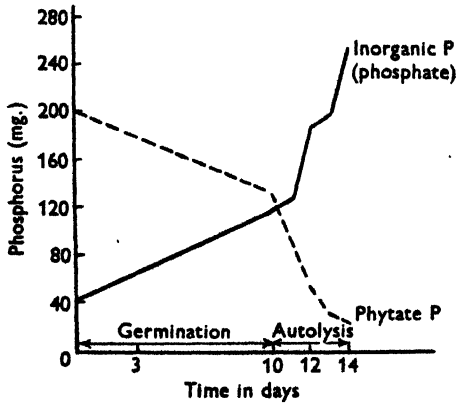
Fig. 2.

Text-fig. 1. Hydrolysis of sodium phytate to inorganic phosphate by the phytase of yeast. Na phytate containing 240 mg. of P was incubated with 5 g. of compressed baker's yeast in 240 ml. of water at pH 4·5 and 45° C.

Text-fig. 2. Hydrolysis of phytate by boiling oatmeal and sodium phytate respectively with 1% HCl. (Composite graph.)

Early in the 1940's an investigation into the hydrolysis of phytate phosphorus during the baking of bread was made and some quantitative data were obtained which proved useful in the later animal experiments relating to the anti-calcifying effect of phytate. For instance, it was found that the yeast was a good source of phytase (Mellanby, 1944). (The effect of incubating a solution of sodium phytate with yeast at pH 4·5 and a temperature of 45° C. can be seen in Text-fig. 1.) McCance & Widdowson (1942*b*), however, used the phytase in bran to hydrolyse the phytate in flour of high extraction in their experiments on human beings.

Examples of the relative rates of hydrolysis of phytate when oatmeal and a solution of sodium phytate respectively are boiled with 1 % HCl are given in Text-fig. 2. The effect on the phytate of germinating oats and then mincing and leaving them to autolyse for a few days at a temperature of about 23° C, is shown in Text-fig. 3, and it will be seen that during germination hydrolysis is slow, but that during the period of autolysis following the mincing of the germinated oats there is a rapid change of the phytate to inorganic phosphate. The changes in the phytate content of oats during germination and autolysis are in line with the effects on the calcification of the teeth brought about by diets containing oats treated in these ways and referred to above (M. Mellanby).



Text-fig. 3. Hydrolysis of phytate of oats during germination and autolysis. The rate of phytate hydrolysis was slow during germination but increased when the germinated grain was minced and allowed to autolyse.

With this later knowledge at hand, some of the earlier experiments were repeated, and in most cases it was found that the cereal preparations with a high phytate content resulted in worse rickets than those in which this substance had been largely converted to inorganic phosphate either by treatment with acid or by a phytase. Sometimes, however, in the later stages the animals receiving the phosphate developed the condition even more quickly than those receiving phytate, so that at the end of the experiment the degree of rickets might differ little in the two cases.

These irregularities in what appeared to be closely comparable experiments were perplexing. With a view to clarifying the position, therefore, all the many earlier investigations made on the relative anti-calcifying properties of hydrolysed and unhydrolysed cereals were reviewed to see what possible differences there were to account for the apparent discrepancies, and it seemed that the vitamin D reserves of the puppies at the beginning of an experiment and the length of time taken to exhaust them might be important factors. For instance, when the reserves were relatively large, owing to the ingestion of cod-liver oil or some other source of vitamin D during the pre-experimental period, then in comparatively short experiments the rickets appeared to be worse in the animals eating the untreated cereals than in those having the treated, but when the reserves were small and were presumably lost more quickly, then differentiation between the two groups did not seem to be nearly so obvious, and in longer experiments was negligible or absent.

The following experiments are representative of the many made with untreated and treated cereals. (For methods of preparation, etc., see below.) They indicate that the inconsistencies above described were related to variations in the amounts of vitamin D in the body when the animals were put on diet, and to the length of time taken for these reserves to be exhausted).

EXPERIMENTAL METHODS

Diets

Most of the litters used in this work were bred at the laboratories from bitches maintained on diets composed of ordinary foodstuffs, including cereals, whole-milk powder, scrap meat and cod-liver oil. During gestation and lactation the dietary Ca was increased, usually by the addition of whole-milk powder, so that the mothers had up to 800 mg. of Ca daily from their food and tap water.

The litters were given food independently of the mother from the 3rd or 4th week after birth, the diet until weaning, which was complete at 6 weeks of age, being of the following form: bread, separated milk powder (up to 25 g.), lean meat (up to 15 g.), compressed baker's yeast, NaCl, ascorbic acid and either peanut oil or cod-liver oil (see individual experiments). This diet was usually continued until the experimental diet was begun.

Full details of the experimental diets are given in the text, but the following general notes may be added here:

(1) The white flour was specially milled and no Ca, phosphorus or preservative was added. There was little or no Ca in the water as in most of the experiments it was either distilled or specially softened.

(2) The Ca content of the basal experimental diets was low but was sufficient, when vitamin D was added, to ensure good calcification except when the diet had a high phytate content.

(3) The daily cereal intake increased as the puppies grew and the Ca:P ratio of the diet was therefore reduced as the experiment proceeded, but was comparable for all animals within an experiment unless otherwise arranged. The cereals were cooked in a pressure steamer (½ lb. pressure) for 1½ hr. and the yeast was boiled with water before being added to them.

(4) All visible fat was removed from the meat.

(5) Various sources of vitamin A were used, but in the later experiments it was given only in the form of vitamin A acetate. The source of vitamin D was calciferol (irradiated ergosterol) obtained through the generosity of British Drug Houses and the amounts administered are expressed as international units (i.u.).

Chemical treatment of some diets

Hydrolysis of phytate of oatmeal by boiling with HCl (*Exps.* 1, 2 *and* 5). 750 g. of oatmeal were mixed with dilute HCl so that the final concentration of acid was 1%, heated on a water-bath for 30 min. and then transferred to a heated sand tray. For the next 30–45 min. the mixture was stirred until it began to boil; boiling was then continued on an electric sand-bath under a reflux condenser for the requisite period. The mass was next cooled under running water, the HCl was neutralised with NaOH, and the bulk was made up to 2250 ml.

Sodium phytate and sodium phosphate (*Exps.* 4, 6, 7, 8 *and* 9). Neutral sodium phytate was prepared from commercial phytin by the method described by Harrison & Mellanby (1939). In experiments which were made to compare the effects of sodium phytate and inorganic phosphate, the latter was prepared from sodium phytate by hydrolysis with yeast (p. 2).

A solution of sodium phytate was mixed with a suspension of baker's yeast (25 mg. of P to 1 g. of yeast), the pH was corrected to 4·5 and the mixture was incubated at 45° C. for 12 hr. with constant stirring. At the end of this period the solution was boiled for 5 min., cooled, and the HCl was neutralized with NaOH. This treatment converted all the phytate to inorganic phosphate.

As controls similar solutions and suspensions of Na phytate and yeast were incubated separately as above. The yeast was boiled and when cool was added to the phytate solution, the HCl being neutralized as before. This mixture contained the same amount of P as the previous one, but in the form of phytate, with only a trace of inorganic P.

Preparation of calcium phytate (*Exp.* 10). 4000 ml. of a solution containing 5 mg. of P per ml. as sodium phytate were brought to pH 5·5 with HCl. 1000 ml. of 6% $CaCl_2$ solution, also at pH 5·5, were added. After being stirred briskly for a few minutes the mass was filtered on a Buchner funnel, washed with a small quantity of water and then with 80 or 90% alcohol. The Ca phytate was then spread out to dry at room temperature and the Ca, total P and phytate P estimated.

Assessment of results

The methods of assessing the experimental results included: (1) X-ray examinations, (2) the estimation of the *A*/*R* ratios, and (3) a study of the mineral metabolism balances. In general these findings were supported by the appearance of the animals and the histological examination of the bones.

As regards the X-ray appearances, an attempt has been made to number the stages of rickets from 1 to 10, but it must be pointed out that these numbers, although relative, do not represent absolute degrees. During life the diagnosis was made from X-rays of the left forepaw only, but post-mortem the appearance of the ribs and other bones was considered.

The method of marking and collecting faecal and urine samples is given on p. 494. The collected faecal samples were heated to 100° C. within 9 hr. of collection, dried, weighed, ground and stored for future analysis of Ca, phytate P and total P.

The methods used in the analysis were:

Estimation of phytate phosphorus in foods, faeces and urine was carried out by the method of McCance & Widdowson (1935), with slight modifications, including an increase in the concentration of the ferric chloride solution.

Total phosphorus in foods, faeces and urine was estimated by the method of McCance & Widdowson (1935).

Inorganic phosphorus and the *total and phytate phosphorus after incineration* were estimated colorimetrically by various methods, including those of Briggs (1922), Fiske & Subarrow (1925), and Allen (1940). For the final comparisons a Spekker photoelectric absorptiometer was used.

Calcium in foods, faeces and urine was estimated by various methods, principally those used by Macy (1942), McCance & Widdowson (1942*a*), and for foodstuffs, the Imperial Bureau of Animal Nutrition (1937).

RESULTS

The relative effect of phytate and phosphate with varying bodily vitamin D reserves

The litters used in these experiments received different amounts of vitamin D during the pre-experimental period. When good supplies were given to the mothers during pregnancy and lactation and to the puppies individually during and after weaning, the body reserves of the vitamin at the beginning of the experimental period would be high. When, however, the puppies had no supplies of the vitamin in this period, their reserves would be lower.

A description is given of the effects on the growing bones of various diets largely composed of cereals. When oatmeal or oats, which are rich in phytate (i.e. about 250 and 180 mg. respectively per 100 g.), were used, they were given to some animals with the phytate intact and to others after much of it had been hydrolysed to inorganic phosphate. In other experiments the effects of phytate and phosphate were tested by giving them as additions to diets whose cereal moiety was white flour which, after cooking, contained little phytate P. In each experiment now to be quoted the basal diet was the same for all members of a litter, so that comparable animals received the same amounts and ratio of calcium and phosphorus.

Untreated oatmeal (phosphorus mainly phytate) compared with oatmeal after boiling with 1% HCl *(phytate hydrolysed to phosphate)*

Exps. 1 *and* 2

Puppies 1 and 2 (Exp. 1) each received 5 ml. of cod-liver oil daily for 3½ weeks before being given the experimental diet at the age of 7 weeks and should, therefore, have had good reserves of vitamin D. The second pair, 3 and 4, which were 6 weeks old at the beginning (Exp. 2), had no cod-liver oil or other source of the vitamin in the pre-experimental period and their reserves of this sub-

TABLE 1 (Exp. 1)

		Dietary conditions					Bone results			
			Phosphorus content of oatmeal eaten (mg./day)					Rickets as judged by X-rays†		
			Phytate		Phosphate*					
No. of puppy	Vitamin D reserves at beginning of exp.	Oatmeal boiled with 1% HCl (hr.)	Min.	Max.	Min.	Max.	*A/R* ratio of femur shaft	After 6 weeks on diet	After 8 weeks on diet	After 11 weeks on diet (P.M.)‡
1	High	0	222	415	118	222	1·00	3	6	8
2	High	18	54	100	286	537	1·37	0	0	1

* Included any organic phosphorus which did not react as phytic acid phosphorus.
† Rickets graded 1–10, the number increasing with the severity of the disease.
‡ P.M. = post-mortem.

Table 2 (Exp. 2)

		Dietary conditions					Bone results			
			Phosphorus content of oatmeal eaten (mg./day)					Rickets as judged by X-rays†		
			Phytate		Phosphate*					
No. of puppy	Vitamin D reserves at beginning of exp.	Oatmeal boiled with 1% HCl (hr.)	Min.	Max.	Min.	Max.	*A/R* ratio of femur shaft	After 6 weeks on diet	After 8 weeks on diet	After 10 weeks on diet (P.M.)
3	Low	0	63	167	50	133	0·97	6	7	8
4	Low	18	18	48	95	252	1·15	1	5	6

* Included any organic phosphorus which did not react as phytic acid phosphorus.
† Rickets graded 1–10, the number increasing with the severity of the disease.

stance would therefore be lower than those of 1 and 2. All had a similar basal diet which was deficient in vitamin D, but one of each pair (1 and 3) had untreated oatmeal whilst the other (2 and 4) received oatmeal which had been boiled with 1% HCl for 18 hr. to hydrolyse its phytate to inorganic phosphate. (About 70% of the phytate was hydrolysed; see Text-fig. 2.)

After 6 weeks each of the animals getting untreated oatmeal (1 and 3) had developed worse rickets than its litter-mate receiving treated oatmeal (2 and 4). This differentiation was maintained between animals 1 and 2 (high vitamin D reserves) for the remainder of the 11-week experimental period (Pl. 1*a*, *b*). In puppies 3 and 4 (relatively low vitamin reserves), however, the differentiation was disappearing at 8 weeks (Pl. 1 (cf. *c* and *d* with *e* and *f*).

These results, typical of the older experiments, supported the suggestion that the differences between the rickets-producing effects of untreated oatmeal (phosphorus present as phytate) and treated oatmeal (phytate hydrolysed to phosphate) were modified by differences in the bodily stores of vitamin D at the beginning of the experiment.

In these early experiments attention was focused on the condition of the bones, as judged by X-ray examination at the beginning and during the course of the experiments and by determining the mineral ash content and the *A*/*R* ratio of one or more bones at the end of the period. As will be seen in discussing Exp. 9, it was later found that, although the *A*/*R* ratio was a good indication of bone quality, it did not necessarily reflect the relative amounts of Ca absorbed from the intestines of the different animals. Although this was not realised at the time of these earlier experiments, it nevertheless seemed essential at this stage to extend the investigation to the study of the absorption of Ca and P from the intestines and the excretion of these substances in both the faeces and the urine under the varying conditions, for it was likely that any difference there might be between the effects of phytate and phosphate on calcium and phosphorus metabolism would be at least partially, and possibly wholly, shown in the alimentary canal. Thus in all the experiments to be described both the end results as regards bone calcification and the course of Ca and P balances during the experimental period were investigated. In the case of P metabolism, both phytate and total phosphorus were determined. The animals were put at intervals of 2–4 weeks in metabolism cages for periods of 2 and later 3 days, the faecal excretion of the period being marked at the beginning and the end by giving carmine in the food and so colouring the stools. The total urine passed during a timed 2- or 3-day period was also collected. Any dogs suspected of eating excreta were muzzled when in the metabolism cages and care was taken to ensure as prompt removal as possible of faeces from the cages. This was usually carried out without difficulty, but in some cases, due to loose stools, shaggy coats, or excessive activity of the dogs, there must have been some loss. When severe rickets occurred it was sometimes impossible to obtain true samples towards the end of the experiment, partly because the animals did not always eat their food readily and partly because of their immobility. In most of these cases the metabolic tests were perforce stopped, although they might be continued for other members of the litter. For these reasons the curves representing Ca and P balances must be considered as a whole and too much emphasis ought not to be placed on individual estimation.

Untreated oats (phytate) compared with germinated and autolysed oats (phosphate)

The relative effects of untreated (high phytate), as compared with germinated and autolysed oats (high phosphate), also varied with the body reserves of vitamin D and were similar to the effects found in the HCl-treated and untreated oatmeal experiments.

Exp. 3

Pre-experimental diet. The first pair of puppies (5 and 6) had a daily addition of 2·5 ml. of cod-liver oil for 4 weeks prior to the experiment and would have good reserves of vitamin D at the beginning. The second pair (7 and 8) received no additional vitamin D or cod-liver oil and would therefore have lower reserves of the vitamin.

Basal experimental diet. Oats, 20–200 g.; separated milk powder, 30 g.; lean meat, 15 g.; peanut oil, 10 ml.; salt, 1 g.; baker's yeast, 5% of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1000 i.u. (Ca content: initial, 407 mg.; final, 565 mg. P content: initial, 326 mg.; final, 904 mg.)

Variations in treatment of oats

No. of puppy	
5	Ground and boiled (phytate intact)
6	Germinated, minced and autolysed (phytate hydrolysed to phosphate)
7	Ground and boiled (phytate intact)
8	Germinated, minced and autolysed (phytate hydrolysed to phosphate)

Age at beginning of experiment: 6 weeks; duration of experiment: 17 weeks

Radiographs (Pl. 2*a–d*) indicate that when the body had good reserves of vitamin D at the beginning of the experiment, as in puppies 5 and 6 (*a* and *b*), the differentiation between the rickets-producing effects of phytate and phosphate was rather greater than when the reserves were lower, as in puppies 7 and 8 (*c* and *d*). Text-fig. 4, representing the daily calcium retentions of the four puppies, shows that, when they were compared according to their vitamin D reserves, there was but little difference between the phytate and the phosphate animal of each pair up to the sixth week of the experimental feeding, when they were eating only relatively little oats and the amount of phytate consumed was still small. From this point, however, as the phytate of the diet increased there was a division into two groups, the first including puppies 5 and 7 (phytate of oats intact) and the second puppies 6 and 8 (phytate of oats converted to phosphate), the amount of calcium retained by the phosphate pair remaining at a higher level than that retained by the phytate pair. In each pair the puppy which originally had high reserves of vitamin D retained slightly more Ca than that with the medium reserves.

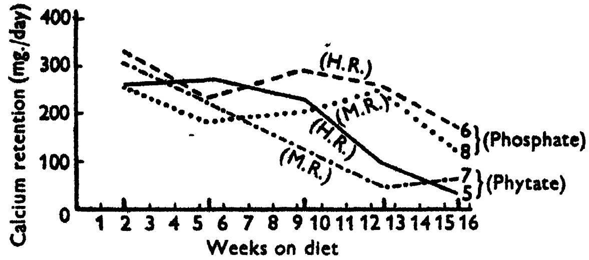
TABLE 3 (Exp. 3)

Dietary conditions

			Phosphorus content of oats eaten (mg./day)				
			Phytate		Phosphate*		Bone results. Rickets as judged by X-rays at P.M.†
No. of puppy	Vitamin D reserves	Treatment of oats	Min.	Max.	Min.	Max.	
5	High	Ground and boiled	28	280	33	330	5
6	High	Germinated, minced and autolysed	0	0	61	610	2
7	Medium	Ground and boiled	28	280	33	330	6
8	Medium	Germinated, minced and autolysed	0	0	61	610	4

* Included any organic phosphorus which did not react as phytic acid phosphorus.

† Rickets graded 1–10, the number increasing with the severity of the disease.



Balance tests on dates marked ⊥.

Text-fig. 4 (Exp. 3). Relative effects of untreated and of germinated and autolysed oats on calcium retention in the presence of high and medium reserves of vitamin D. *Note.* In the presence of both high (H.R.) and medium (M.R.) vitamin D reserves the Ca retention of puppies 5 and 7, having untreated oats (phytate), was lower than that of puppies 6 and 8, receiving germinated, minced and autolysed oats (phytate converted to phosphate).

In view of the above results, from which it appeared that phytate in oatmeal and oats lost some of its rachitogenic effect after hydrolysis to inorganic phosphate and that the relative effects of these two forms of P in the diet were in some way determined by the presence in or absence from the body of vitamin D, it was decided to make a more complete study of this problem. Instead of giving cereals containing phytate, this substance was prepared as the neutral sodium salt and given to puppies either as such or, after hydrolysis by yeast, as inorganic phosphate. It thus became possible to get a clearer view of the relative action of these substances on calcification uncomplicated by other unknown dietetic factors.

Sodium phytate prepared from commercial phytin compared with sodium phosphate

The object of Exp. 4 was to see in the first place whether the results obtained in Exps. 1–3, in which treated and untreated oatmeal and oats were the variable factors, could be repeated with phytate and inorganic phosphate. It was also desired to determine whether there was a difference in the amounts of Ca and P absorbed from the gut when these two salts were used and, if so, whether this difference persisted when the vitamin D reserves were exhausted. The experimental diets did not contain vitamin D and the pre-experimental diets were so arranged that two of the four animals would have high and two low D reserves. Substantial differences in calcium retention would thus be expected, the animals having good initial reserves of the vitamin being likely to retain more calcium than the corresponding low-reserve animals.

Exp. 4

Pre-experimental diet. From the age of 4–7 weeks puppies 9 and 10 were each given 500 i.u. vitamin D_2 daily. It was certain, therefore, that both had substantial stores of the vitamin at the beginning of the experiment. Puppies 11 and 12, on the other hand, although receiving the same low D basal diet, had no vitamin D_2 supplement during the same period, so that their stores were smaller.

Basal experimental diet. White flour, 35–145 g.; separated milk powder, 30 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 1% of cereal; baker's yeast, 10% of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1000 i.u. (Ca content: initial, 406 mg.; final, 421 mg. P content: initial, 301 mg.; final, 393 mg.)

Daily additions to basal diet

No. of puppy	Mg. phytate *P*/100 g. flour	Mg. phosphate *P*/100 g. flour
9	250	10
10	0	260
11	250	10
12	0	260

Age at beginning of experiment: 7 weeks; duration of experiment: 23–24 weeks

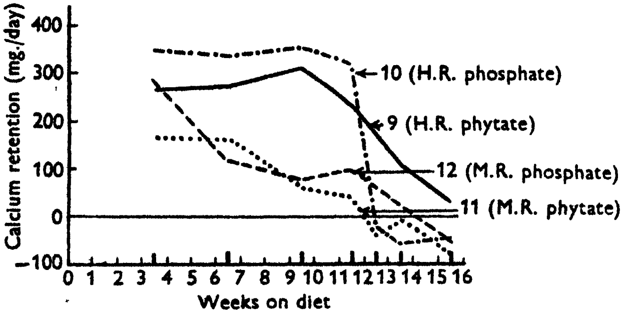
All four puppies received the same total amount of phosphorus daily, but the quantity given to 10 and 12 in excess of that present in the basal diet was in the inorganic form, whereas the extra given to 9 and 11 was mainly phytate phosphorus. It will be seen from Text-fig. 5 that the calcium retention of 9 and 10, both of which had a comparatively large store of vitamin D at the beginning of the experiment, remained high for the first 9 weeks. There was, however, during this period a real difference between the amount retained by the two animals, 10 (receiving P as phosphate) having a consistently higher retention than 9 (phytate). After 11 weeks of the diet it would appear that the vitamin D stores of these two puppies were greatly reduced and the power to retain calcium was rapidly lost. In puppies 11 and 12, whose vitamin D reserves were known to be smaller at the beginning of the experimental period, the Ca retention soon began to fall and there was little difference between the absorption of Ca in the phytate and phosphate animals after 4–6 weeks.

The radiographs of these animals showed that the high vitamin D reserves of puppies 9 and 10 were sufficient to protect them against rickets until the thirteenth week of the experiment, at which time 11 and 12, which had smaller reserves, both had severe rickets. At the end of the experimental period (i.e. after approximately 24 weeks) the disease had developed in the previously rickets-free pair but to a less severe extent. In both pairs the puppy receiving phytate was more rachitic than that receiving phosphate, but in the pair with only moderate vitamin D reserves the early definite differentiation was gradually reduced.

TABLE 4 (Exp. 4)

		Dietary conditions. Phosphorus added to basal diet (mg./day)				Bone results. Rickets as judged by X-rays†		
		Na phytate		Inorganic phosphate*				
No. of puppy	Vitamin D reserves	Min.	Max.	Min.	Max.	After 10 weeks on diet	After 13 weeks on diet	At P.M.
9	High	88	363	3	14	0	0	3
10	High	0	0	91	377	0	0	2
11	Medium	88	363	3	14	8	9	10
12	Medium	0	0	91	377	4	6	8

* Na phytate hydrolysed by phytase of yeast.
† Rickets graded 1–10, the number increasing with the severity of the disease.



Text-fig. 5 (Exp. 4). Relative effects of phytate and phosphate on calcium retention in the presence of high and medium reserves of vitamin D. *Note.* (1) when the vitamin D reserves were high (H.R.) the Ca retention of puppy 9 (phytate) was lower than that of puppy 10 (phosphate prepared from phytate); (2) when the vitamin D reserves were lower (M.R.), there was but little difference in the Ca retentions of puppies 11 (phytate) and 12 (phosphate prepared from phytate).

The results of this experiment, therefore, lent support to the hypothesis upon the basis of which it was carried out. It suggested that: (1) when vitamin D was present in the body, even when there was none in the food, phytate prevented the absorption and retention of calcium to a greater extent than phosphate of equal phosphorus content; and (2) when the body had no reserves of the vitamin and there was none in the food, calcium absorption was very small in any case, and phosphate was probably as potent as phytate in further reducing its absorption (Mellanby, 1937).

It was next decided to see whether phytate and phosphate, both in oatmeal and as the Na salts prepared from commercial phytin, had the same relative effects on the absorption and utilization of Ca when animals received no vitamin D and 20 i.u. daily respectively.

The relative effect of phytate and phosphate in the presence and absence of dietary vitamin D_2

Untreated oatmeal (phytate) compared with oatmeal hydrolysed by HCl *(phosphate)*

Exp. 5

Pre-experimental diet. When fed independently of the mother, this series of puppies received no cod-liver oil or other source of vitamin D. Thus at the beginning of the experiment there should have been only moderate reserves of the vitamin in all the animals.

Basal experimental diet. Oatmeal, 35–140 g.; separated milk powder, 30 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 4·8% of cereal; baker's yeast, 5% of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1000 i.u. (Ca content: initial, 362 mg.; final, 414 mg. P content: initial, 436 mg.; final, 883 mg.)

Variations in treatment of oatmeal

No. of puppy	
13	Untreated (phytate intact)
14	Boiled with HCl for 18 hr. (most of phytate hydrolysed to phosphate)
15	Untreated (phytate intact)
16	Boiled with HCl for 18 hr. (most of phytate hydrolysed to phosphate)

Daily additions to basal diet

No. of puppy	
13	20 i.u. vitamin D_2
14	20 i.u. vitamin D_2
15	None
16	None

Age at beginning of experiment: 7 weeks; duration of experiment: 15 and 16, 17½ weeks; 13 and 14, 19½ weeks.

In this experiment oatmeal was the source of phytate and phosphate. Although all the animals in the litter received the same total amount of P in the food, it will be seen from Table 5 that the P of 14 and 16 (acid-treated oatmeal) was mostly in the inorganic form, whereas that of 13 and 15 contained much phytate. The Ca balance results are given in Text-fig. 6, which shows that in 15 and 16, neither of which received vitamin D and both of which developed severe rickets (as judged by X-rays), the Ca retention fell throughout the experiment and the phytate reduced the Ca balance to a greater extent than the phosphate. Vitamin D protected puppies 13 and 14 against rickets, but although both maintained a good positive calcium balance, puppy 13, receiving phytate P, had a lower retention than 14, receiving phosphate.

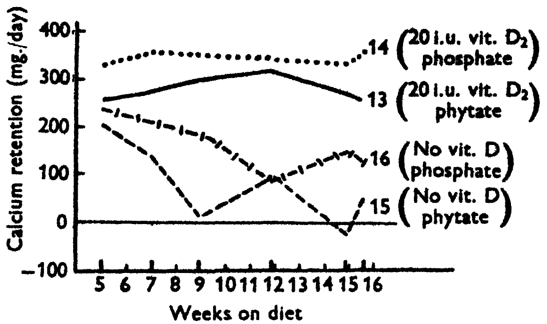
This experiment, therefore, indicated that, even in the presence of sufficient dietary vitamin D_2 to protect the animals against rickets, phytate exerted a greater inhibiting effect on Ca absorption than phosphate, thereby lowering retention, whereas in the absence of the vitamin the absorption of Ca was small and irregular whether phytate or phosphate was given.

TABLE 5 (Exp. 5)

	Dietary conditions						Bone results.
		Phosphorus in oatmeal eaten (mg./day)					
No. of puppy	Oatmeal boiled with 1 % HCl (hr.)	Phytate		Phosphate*		Vitamin D_2 (i.u.)	Rickets as judged by X-rays at P.M.†
		Min.	Max.	Min.	Max.		
13	0	80	380	47	220	20	0
14	18	23	113	104	487	20	0
15	0	80	380	47	220	0	10
16	18	23	113	104	487	0	9

* Included any organic phosphorus which did not react as phytic acid phosphorus.

† Rickets graded 1–10, the number increasing with the severity of the disease.



Text-fig. 6 (Exp. 5). Relative effects of untreated and HCl treated oatmeal on calcium retention in the absence of vitamin D and in the presence of 20 i.u. daily. Untreated oatmeal (phytate) (13 and 15) exerted a greater inhibiting action on Ca absorption than treated oatmeal (phosphate) (14 and 16) both in the presence of dietary vitamin D_2 (13 and 14) and in its absence (15 and 16).

Sodium phytate compared with sodium phosphate

In this experiment, unlike the last, sodium phytate and phosphate respectively were added to the diets, instead of oatmeal being used as a source of phytate and acid-treated oatmeal as a source of phosphate.

Exp. 6

Pre-experimental diet. The mother of the litter received cod-liver oil during pregnancy and the first 4 weeks of lactation, but the diet of the puppies after weaning contained no cod-liver oil or other form of vitamin D.

Basal experimental diet. White flour, 25–160 g.; separated milk powder, 30 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 1 % of cereal; baker's yeast, 10 % of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1000 i.u. (Ca content: initial, 390 mg.; final, 434 mg. P content: initial, 296 mg.; final 450 mg.)

Daily additions to basal diet

No. of puppy	Mg. phytate P/100 g. flour	Mg. phosphate P/100 g. flour	Vitamin D_2 (i.u.)
17	250	10	20
18	0	260	20
19	250	10	0
20	0	260	0

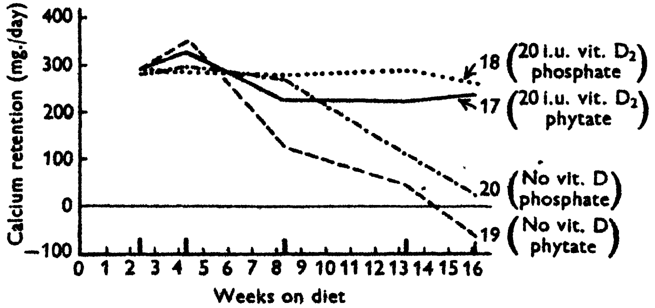
Age at beginning of experiment: 6½ weeks; duration of experiment: 23 weeks.

These animals were periodically tested for calcium and phosphorus retention and the results are given in Text-fig. 7. It will be seen that the addition of vitamin D_2 to the diet improved the calcium retention of both phytate and phosphate animals. Phytate, however, still exerted an inhibiting effect on Ca retention and, as the figure shows, both with and without the addition of vitamin D to the diet phosphate allowed a higher Ca retention than phytate.

TABLE 6 (Exp. 6)

	Dietary conditions					Bone results.
	Phosphorus added to basal diet (mg./day)					
	Na phytate		Inorganic phosphate*			
No. of puppy	Min.	Max.	Min.	Max.	Vitamin D_2 (i.u.)	Rickets as judged by X-rays at P.M.†
17	62	400	3	16	20	0
18	0	0	65	416	20	0
19	62	400	3	16	0	9
20	0	0	65	416	0	9

* Na phytate hydrolysed by phytase of yeast.
† Rickets graded 1–10, the number increasing with the severity of the disease.



Text-fig. 7 (Exp. 6). Relative effects of phytate and phosphate on Ca retention in the absence of vitamin D and in the presence of 20 i.u. daily. *Note.* (1) In the presence of dietary vitamin D_2 retention of Ca was lower with phytate (17) than with phosphate (18); (2) in the absence of dietary vitamin D_2 the power to retain Ca was soon reduced in both animals irrespective of the form of P ingested [phytate animal (19) about 2 weeks before phosphate (20)].

In all the foregoing experiments the dietary Ca : P ratio was low at the beginning and fell during the course of the feeding period as the consumption of cereal increased. Although the amounts and ratios of Ca and P varied from experiment to experiment they did not vary within an experiment, being the same for all the puppies of a litter at any given time. The form of the phosphorus, however, varied; sometimes it was present as inorganic phosphate and sometimes partly as phytate.

The results of Exps. 4–6 showed that:

(1) In the presence of vitamin D, either in the diet or in the body as reserves, phytate reduced Ca absorption from the gut more than did inorganic phosphate of equal P content.

(2) As the vitamin D stores of the body were used up, Ca absorption was always greatly diminished in the presence of either phytate or phosphate, phytate usually, though not in all cases, proving the more powerful in this respect.

(3) So far as was indicated by Ca retention and radiographs of the bones, the effects of giving a high phytate-containing cereal such as oatmeal, before and after treatment which converted much of its phytate to inorganic phosphate, could be repeated by adding sodium phytate and sodium phosphate respectively to diets of low phytate content.

The time and rate at which the Ca retention of D-deficient animals fell varied from experiment to experiment. This irregularity was largely due to differences in and rates of loss of body stores of the vitamin and could be roughly controlled by regulating the vitamin D intake in the pre-experimental period.

Having demonstrated that, in the presence of vitamin D, phytate had a specific action in reducing Ca absorption, it now seemed desirable to compare the effect of adding phytate and phosphate respectively to diets of relatively low P content. The previous experiments, owing to their form, had not disclosed whether inorganic phosphate, in the amounts given, had any depressing effect on Ca absorption from the gut, but results suggested that, when there was sufficient vitamin D available, additional phosphate had comparatively little effect, whilst phytate definitely caused a large reduction in the Ca absorbed; on the other hand, when the vitamin reserves were depleted the two substances probably had more equal depressing effects on Ca absorption.

The next experiment was designed to test this point. Two puppies were given diets of a lower Ca : P ratio than the control, one receiving an addition of P as phytate and the other an equal amount as phosphate. All three animals had a trace of dietary vitamin D_2 to supplement the body reserves but not sufficient to prevent rickets.

Effect of increasing the dietary P by additions of phytate and phosphate on calcium retention

Exp. 7

Pre-experimental diet. When the puppies were fed separately from the mother they received no cod-liver oil or other source of vitamin D.

Basal experimental diet. White flour, 20–160 g.; separated milk powder, 20 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 2 g.; baker's yeast, 10% of cereal; ascorbic acid, 5 mg.; vitamin A acetate 1500 i.u.

(Ca content: initial, 267 mg.; final, 292 mg. P content: initial, 230 mg.; final, 410 mg.)

Daily additions to basal diet

No. of puppy	Mg. P as Na phytate/100 g. flour	Mg. P as Na phosphate/100g. flour	Vitamin D_2 (i.u.)
21	250	10	2
22	0	260	2
23	0	0	2

Age at beginning of experiment: 6½ weeks; duration of experiment: 15½ weeks.

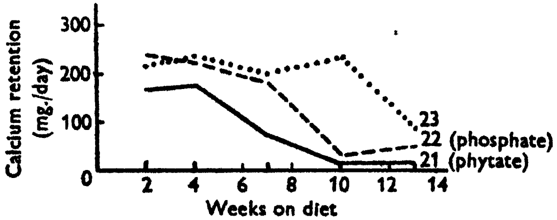
Text-fig. 8 shows that during the early part of the experiment, when all three animals had some reserves of vitamin D, the addition to the diet of phosphate (22), and incidentally the reduction of the Ca:P ratio, had but little effect on Ca retention, whereas the addition of the same amount of P as phytate (21) and the same alteration in the Ca:P ratio had a definite effect. After 10 weeks of the experiment the differentiation between puppies 21 and 22 was disappearing, phytate and phosphate then behaving similarly in that both reduced the Ca retention. After a further 3 weeks, when the last metabolic test was made, all the animals had greatly reduced powers of Ca absorption, but the two receiving diets with the lower Ca:P ratios were still retaining less than the control animal (23).

TABLE 7 (Exp. 7)

	Dietary conditions					Bone results		
	Phosphorus added to basal diet (mg./day)					Rickets as judged by X-rays at†		
	Na phytate		Na phosphate*					
No. of puppy	Min.	Max.	Min.	Max.	Vitamin D_2 (i.u.)	7 weeks	11 weeks	P.M.
21	50	400	2	16	2	2	6	8
22	0	0	52	416	2	1	3	5
23	0	0	0	0	2	0	1	3

* Na phytate hydrolysed by phytase of yeast.

† Rickets graded 1–10, the number increasing with the severity of the disease.



Text-fig. 8 (Exp. 7). Relative effects of reducing the Ca:P ratio of the diet by additions of phytate or phosphate P on Ca retention. *Note.* (1) When, in addition to a trace (2 i.u. daily) of dietary vitamin D_2 there were also body reserves, lowering the Ca:P ratio by the addition of phytate (21) reduced Ca retention to a greater extent than a similar change in the ratio brought about by phosphate (22); (2) later (after 10 weeks) when the vitamin D reserves were depleted the effects of the two forms of P were more alike; (3) the addition of P in either form appeared to hasten the loss of vitamin D reserves, as indicated by diminution of Ca absorption, and phytate probably did this to a greater extent than phosphate.

Text-fig. 8 also shows that the 2 i.u. of vitamin D given daily to each of the animals were not sufficient either to maintain good calcium absorption or even to conserve the body reserves of the vitamin. The Ca retention curves of the three animals fell sharply at different times of the experimental feeding. Thus the control animal (23) began to lose its power to retain Ca after 10 weeks, the phosphate animal (22) after 7 weeks and the phytate animal (21) after 4 weeks. It would appear that the addition of either type of P hastened the removal of stored vitamin D and that phytate did so more rapidly than phosphate.

X-ray examination of the bones showed that after 7 weeks of the experimental feeding there was definite rickets in puppy 21 (phytate), whilst 22 (phosphate) was still nearly normal and 23 was without doubt normal from this point of view. From this time onwards the disease developed in the three animals, the two with additional P developing more severe rickets than the control (23), while puppy 21 (phytate) had worse rickets than 22 (inorganic phosphate).

Most of the criticism of phytate as an anti-calcifying agent has been based on rat experiments in which diets with a high-Ca, low-P ratio were used and in which P was without doubt the limiting factor as regards calcification. In such experiments the addition of P to the diet improved both Ca and P absorption and bone calcification. In the present experiment, however, where Ca was the limiting factor, additions of P both as phytate and phosphate reduced Ca absorption, as has been shown above, but resulted in an increased absorption of P. Phosphate, which had the smaller depressing effect on Ca absorption, increased the P absorption to a greater extent than phytate. Thus after 4 weeks, when there were still body reserves of vitamin D, the addition of phytate increased P absorption from 267 (23) to 393 mg. (21) daily, and the addition of phosphate increased it from 267 (23) to 495 mg. (22) daily. Later (13 weeks), when the vitamin reserves were very low, the P absorptions were 191 mg. (23, no extra P), 385 mg. (21, phytate), and 589 mg. (22, phosphate). Most of the additional P absorbed from the high P diets was excreted via the urine so that the amounts of P retained by the three animals were approximately the same.

This experiment therefore indicates that:

(1) Since 2 i.u. of vitamin D daily do not prevent the depletion of this vitamin, and all three animals so fed lost the power to maintain Ca absorption.

(2) Adding equal amounts of phytate and phosphate, respectively, to the diets of litter-mate puppies, and thereby lowering the Ca:P ratio of their food to the same extent, has widely different effects on their Ca retention as long as sufficient vitamin D is available.

(3) The addition of phytate to the diet hastens the onset of rickets and increases its severity to a greater degree than the addition of an equal amount of P as phosphate.

(4) The addition of either type of P appears to hasten the exhaustion of body reserves of vitamin D, but phytate P probably acts more strongly than an equal amount of P as phosphate.

(5) Increasing the dietary P by additions of phytate or phosphate increases P absorption under these conditions, and even when the vitamin D supply is insufficient, the absorption of P is increased to a greater extent by phosphate than by phytate.

Exp. 7 shows quite clearly that the Ca: P ratio is not a reliable indication of the calcifying qualities of a diet. It is true that, when the dietary supply of vitamin D was very low and the body reserves were depleted, lowering the Ca: P ratio by additions of P reduced Ca absorption. On the other hand, during the early part of the experiment, when there was a sufficiency of vitamin D, there was a distinct difference in the results produced by altering the ratio to the same extent by the addition of phytate and phosphate respectively. This difference was not due to unavailable P of the phytate reducing the need for Ca, for puppy 21 (phytate) absorbed more P, some of which must have come from the phytate, than 23 (no addition), although the Ca absorption was much reduced. Thus some of the P fed as phytate was available for absorption and it seems probable that it was only the remainder which, combining with Ca in the gut and thereby removing it from the influence of absorptive processes, reduced Ca absorption (Harrison & Mellanby, 1939).

These experiments on dogs demonstrated that the calcifying qualities of a diet could only be judged when a number of factors, including the presence or absence of vitamin D, the total amounts of Ca and P in the diet and the form of the P, were known. The form of the dietary P appeared in these experiments to be a crucial factor and it now seemed desirable to find out how phytate, as compared with phosphate, decreased Ca absorption and whether, in fact, there was a quantitative relationship between the excretion of these two substances, so that an increase in phytate excretion brought with it an increase in Ca excretion. In the next experiment, therefore, whilst keeping the total dietary P and calcium equal, three different levels of phytate P were given in order to test the possibility of this relationship.

The effect of increasing the dietary phytate but not the total phosphorus on calcium and phytate excretion

Exp. 8

The pre-experimental diet of the puppies contained no cod-liver oil or other source of vitamin D.

Basal experimental diet. White flour, 20–160 g.; separated milk powder, 20 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 1 % of cereal; baker's yeast, 12 % of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1500 i.u. (Ca content: initial, 265 mg.; final, 297 mg. P content: initial, 209 mg.; final 377 mg.)

Daily additions to basal diet

No. of puppy	Mg. phytate P/100 g. flour	Mg. phosphate P*/100 g. flour	Vitamin D_2 (i.u.)
24	300	18	20
25	150	168	20
26	0	318	20

* Na phytate hydrolysed by phytase of yeast.

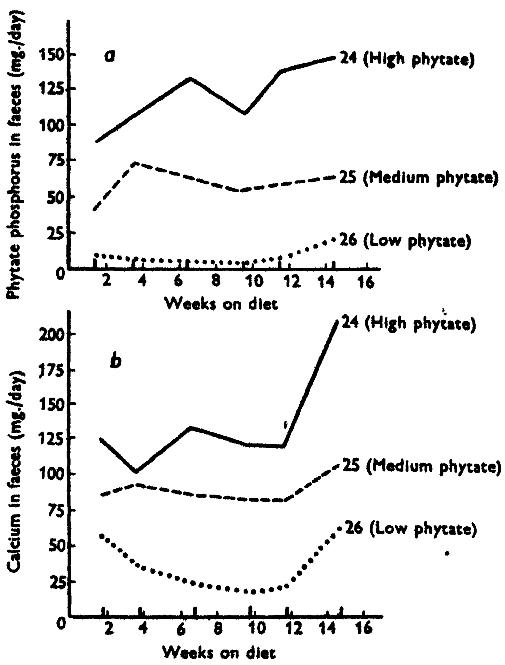
Age at beginning of experiment: 6½ weeks; duration of experiment: 16½ weeks.

The effect of the different diets tested in this experiment on the phytate and Ca in the faeces is shown in Text-fig. 9*a*, *b*. The amount of dietary phytate varied greatly (high in puppy 24, low in 26 and intermediate in 25), and it was found that the unabsorbed phytate was related to the intake (Text-fig. 9*a*).

Table 8 (Exp. 8)

	Dietary conditions				
	Phosphorus added to basal diet (mg./day)				
	Na phytate		Na phosphate*		
No. of puppy	Min.	Max.	Min.	Max.	Vitamin D_2 (i.u.)
24	60	480	4	29	20
25	30	240	34	269	20
26	0	0	64	509	20

* Na phytate hydrolysed by phytase of yeast.



Text-fig. 9 (Exp. 8). Effect of increasing the phytate P, but not the total P of the diet, on the faecal excretion of phytate and Ca. (*a*) In the presence of vitamin D, increasing the phytate of the diet when the total P intake was kept constant raised the faecal phytate; (*b*) in the presence of vitamin D increasing the phytate of the diet when the total P intake was kept constant raised the faecal calcium.

Although the Ca, P and vitamin D content of the food of these animals was identical, a high-phytate low-phosphate intake was accompanied not only by a high phytate but also by a high Ca excretion (Text-fig. 9*b*), a low-phytate high-phosphate intake by both a low phytate and low Ca excretion, whilst an intermediate phytate intake resulted in an intermediate Ca excretion. It is evident, therefore, that under the conditions of this experiment the amount of phytate in the diet assumed a position of great importance. Text-fig. 9*a* leaves no doubt as to the Ca-depriving action of phytate as compared with phosphate of the same P content, when vitamin D was available to the animal.

Comparison of the Ca excretions of puppies 24, 25 and 26 after only 2 weeks of the experimental period, when the total daily intake of phosphorus was 451 mg., shows that an increase in the phytate P from 21 mg. in the diet of puppy 26 to 111 mg. in that of 25, and from 111 mg. in the diet of 25 to 201 mg. in that of 24, produced in both instances about a 50 % increase in Ca excretion.

Only three animals have been described in this experiment, although tests were carried out on six. The group not mentioned above received the same amounts and forms of P and Ca, but the vitamin D intake was 100 i.u. instead of 20 i.u. daily. This increase in vitamin D supplies did not appear to modify the Ca-depriving effect of phytate in this instance. In the next experiment, therefore, the effect of giving 20, 100 and 1000 i.u. respectively of vitamin D daily at two levels of phytate intake was determined.

The relative effect of phytate and phosphate on calcium and phytate excretion with varying quantities of dietary vitamin D_2

Exp. 9 *Sodium phytate and sodium phosphate*

Pre-experimental diet. From the age of 3 weeks each puppy received 2·5 ml. of cod-liver oil daily for 23 days, after which period the oil was discontinued.

Basal experimental diet. White flour, 50–160 g.; separated milk powder, 30–20 g. (reduced after 3 weeks on diet); lean meat, 15 g.; peanut oil, 11 ml.; NaCl, 1 % of cereal; baker's yeast, 12 % of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1500 i.u. (Ca content: initial, 367 mg.; final 293 mg. P content: initial, 305 mg.; final, 372 mg.)

Daily additions to basal diet

No. of puppy	Mg. phytate P/100 g. flour	Mg. phosphate P*/100 g. flour	Vitamin D_2 (i.u.)
27	0	318	20
28	300	18	20
29	0	318	100
30	300	18	100
31	0	318	1000
32	300	18	1000

* Na phytate hydrolysed by phytase of yeast.

Age at beginning of experiment: 7 weeks; duration of experiment: 15½ weeks.

Text-fig. 10*a, b* shows that, as in the previous experiment, a high phytate intake was accompanied by both a high phytate and a high Ca excretion. The point to be noted from these figures is that increasing the vitamin D intake from 20 i.u. in the case of puppies 27 and 28 to 100 i.u. in 29 and 30 and to 1000 i.u. in 31 and 32 did not greatly increase the amount of Ca absorbed (Text-fig. 11) and did not significantly alter the depressing effect of phytate on Ca absorption.

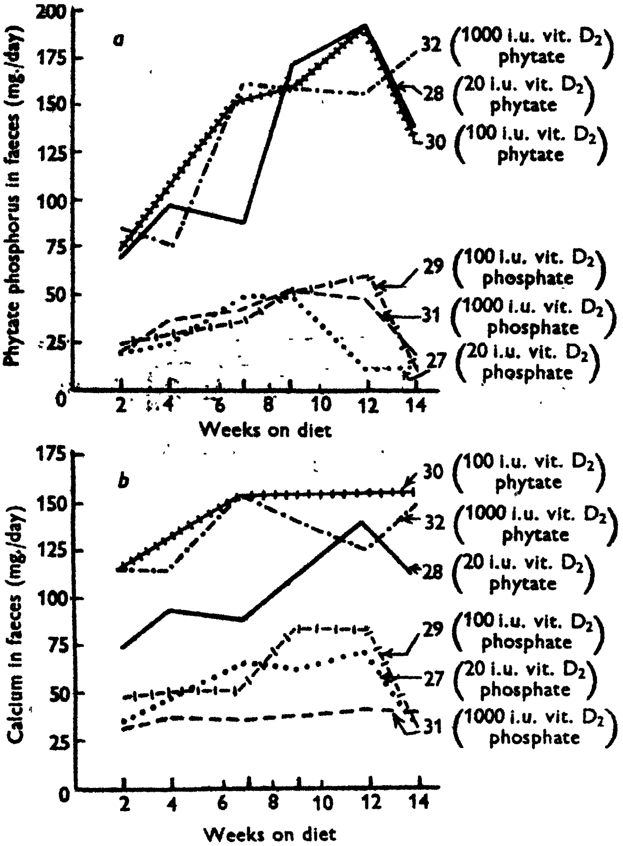
This result on Ca absorption was in marked contrast to the difference observed in Exp. 6 (Text-fig. 7) between an animal having no vitamin D in the food or body and one to which a relatively small amount, 20 i.u. daily, was given. In such a case the presence of vitamin D was the determining factor as regards Ca absorption from the gut. On the other hand, Text-fig. 10*b* shows that under the conditions of Exp. 9 the amount of phytate in the food again assumed a position of great importance and greatly affected the amount of Ca excreted. It will be seen later in Exp. 12, where oatmeal was the source of the dietary phytate, that increasing the vitamin D above 20 i.u. increased Ca absorption under some circumstances. Whether this was due to the fact that the phytate was a constituent of the oatmeal and not, as in Exp. 9, free Na phytate, or whether it was due to other variations between the two experiments is not known. Clearly 20 i.u. of vitamin D_2 daily are near the 'limiting' amount, i.e. the level of intake which produces optimum absorption of Ca from the gut.

TABLE 9 (Exp. 9)

	Dietary conditions					Bone results		
	Phosphorus added to basal diet (mg./day)							
	Na phytate		Na phosphate*					
No. of puppy	Min.	Max.	Min.	Max.	Vitamin D_2 (i.u.)	Combined weight of ash of humerus radius and ulna (g.)	Combined weight of ash of 5th, 6th 7th and 8th ribs (g.)	*A/R* ratio of femur shaft
27	0	0	159	509	20	6·555	0·987	1·13
28	150	480	9	29	20	5·762	0·678	0·87
29	0	0	159	509	100	6·449	0·958	1·25
30	150	480	9	29	100	5·053	0·724	1·07
31	0	0	159	509	1000	6·732	0·929	1·26
32	150	480	9	29	1000	5·385	0·695	1·19

* Na phytate hydrolysed by phytase of yeast.

Whilst little or no rickets could be produced under the conditions of Exp. 9 because of the presence of vitamin D, the effect of phytate would be to reduce the amount of Ca in the bones. This can be seen to have happened, since the average ash weight of the humerus, radius and ulna of the three phytate animals was 5·4 g. and that of the corresponding bones of the phosphate animals 6·579 g. Thus, the relative effects of phytate and phosphate on the absorption of Ca from the alimentary canal (as seen in Text-fig. 11) were reflected in the amount of calcium in the bones. It has also been seen that variations in the daily amount of vitamin D did not cause significant differences in the amount of Ca absorbed from the gut, either in the phytate or in the phosphate animals. In keeping with this result, it will also be noticed that the combined mineral ash of the humerus, radius and ulna was similar, namely 6·555, 6·449, and 6·732 g. in the phosphate animals and 5·762, 5·053 and 5·385 g. in the three animals taking phytate. Thus, the amount of Ca salts deposited in the bones, while greatly affected by phytate as compared with phosphate in the food, was not significantly affected by increasing the amount of dietary vitamin D above a certain quantity.

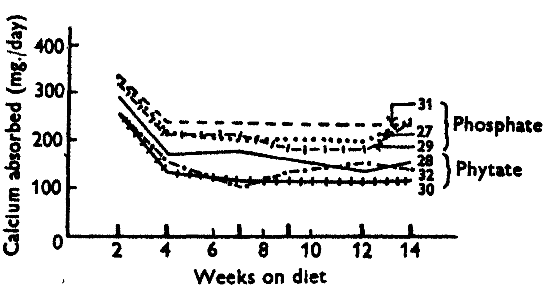


Text-fig. 10 (Exp. 9). Effects of increasing the dietary vitamin D_2 on the faecal excretion of phytate and Ca.

No. of puppy	Vitamin D_2 (i.u.)	
27	20	Phosphate
28	20	Phytate
29	100	Phosphate
30	100	Phytate
31	1000	Phosphate
32	1000	Phytate

Note. (1) Increasing the dietary phytate at the expense of phosphate increased both the phytate (*a*) and to some extent the Ca (*b*) in the faeces; (2) a large increase of vitamin D_2, i.e. from 20 to 1000 i.u. daily, did not significantly alter the effect of the dietary phytate on phytate (*a*) and Ca (*b*) excretion.

When, however, the *A*/*R* ratios of the femurs are considered (Table 9), it is evident that increasing the dietary vitamin D caused an improvement in the quality of the bone formed. This was especially the case in the phytate animals, where the *A*/*R* ratios were 0·87 (20 i.u. vitamin D), 1·07 (100 i.u.) and 1·19 (1000 i.u.). In comparison the phosphate animals showed a relatively small improvement in these ratios with increasing doses of vitamin D. Thus although increasing the vitamin D in the presence of phytate had no effect on the amount of Ca deposited in the bones, it caused an improvement in the structure of the bones which was indicated by the better *A*/*R* ratios. The same relationship was found in the ribs. Pl. 2 (*e*–*g*) shows the radiographic appearance of the costochondral junctions of 27, 28 and 32, and it will be seen that, in the presence of 20 i.u. of vitamin D_2, a diet in which the P was mainly phosphate (*e*) produced well-formed bones, whereas when the phosphate was largely replaced by phytate of equal P content, the bones were thick and osteoporotic (*f*). Thus, in the presence of equal dietary vitamin D, replacing phosphate by phytate lowered Ca absorption (Text-fig. 11), reduced the mineral content of the ribs (Table 9) and, as would be expected, reduced the quality of the bones (Pl. 2*e*,*f*).



Text-fig. 11 (Exp. 9). Relative effects of phytate and phosphate on calcium absorption in the presence of different amounts of vitamin D_2.

No. of puppy	Vitamin D_2 (i.u.)	
27	20	Phosphate
28	20	Phytate
29	100	Phosphate
30	100	Phytate
31	1000	Phosphate
32	1000	Phytate

Note. Increasing the vitamin D_2 intake fifty-fold (from 20 to 1000 i.u. daily) did not appear to influence the effect of phytate in reducing Ca absorption.

The effect of raising the vitamin D intake from 20 to 1000 i.u. daily (*g*) with equal dietary phytate was more interesting, for it greatly improved the structure of the bone (cf. *f* and *g*) without significantly altering the Ca absorption (Text-fig. 11) or the mineral ash of the bone (Table 9), and the thin compact bones of puppy 32 (*g*) were comparable in radiographic appearance to those of puppy 27 (*e*).

It is obvious, therefore, that, when phytate forms a substantial part of the diet, raising the vitamin D intake, although not ensuring a higher Ca absorption or higher mineral content of the bones, will produce better calcified and more compact bones.

Note In the course of this work it became clear that the *A*/*R* ratio (Chick, Korenchevsky & Roscoe, 1926; Chick & Roscoe, 1926) has only a limited usefulness as a measure of calcium retention. In this ratio, *A* is the weight of the ash of the bone and *R* the weight of the dried, fat-extracted bone less the weight of the ash. Thus the *A*/*R* ratio becomes a measure of the proportion of the mineral ash content of the bone to its dried, fat-free organic tissues. Obviously this ratio may be varied by alterations in the weight of mineral ash when the organic tissue is constant or, in the case of constant amounts of mineral ash, by alterations in the amount of organic tissue. When therefore it became apparent that alterations in vitamin D supplies might vary the *A*/*R* ratio and not the ash content (cf. puppies 28 and 32, Table 9), the ratio as a measure of calcification was abandoned.

In Exp. 8 the close relationship between phytate and Ca in the faeces was seen, a rise or fall in the excretion of phytate being associated with a similar change in the excretion of Ca. Exp. 9 showed that large increases in vitamin D could not, under the conditions of that experiment, overcome the depressing effect of phytate on Ca absorption. It seemed likely that these unabsorbed substances were associated in the faeces, probably as an insoluble Ca phytate, and that when this combination took place in the intestine the Ca became unavailable to the animal. It was therefore of interest to determine whether the animal could make use of Ca phytate if the substance were presented in the food in an insoluble form and, if so, how the availability of this substance compared with that of Ca phosphate. It was probable that there would be some breakdown of each in the stomach, with the formation of the soluble $CaCl_2$. On the other hand, there was an equal possibility that in the duodenum the Ca might again react with the phytate and return to its less soluble form. In the following experiment practically all the Ca of the diet was given in the form of either penta-calcium phytate or Ca phosphate; on the other hand, only about 70% of the total P intake came from the Ca phytate. The object was to see to what extent dogs could make use of the Ca and P of these compounds and whether a large increase of vitamin D in the diet facilitated their absorption.

Calcium phytate or calcium phosphate as source of dietary calcium

Exp. 10

The pre-experimental diet of the puppies contained no cod-liver oil for 3½ weeks prior to the beginning of the experimental period.

Basal experimental diet. White flour, 80–120 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 1 g.; baker's yeast, 5% of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1500 i.u. (Ca content: initial 22 mg.; final, 30 mg. P content: initial, 125 mg.; final, 175 mg.)

Note. No separated milk powder; hence Ca of diet low.

Daily additions to basal diet

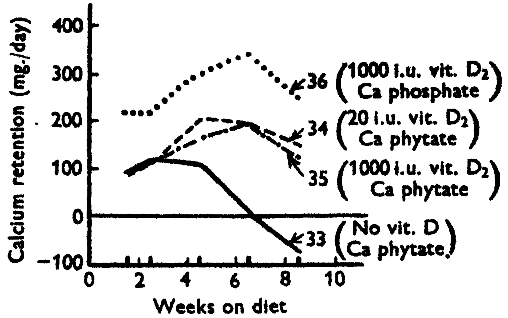
No. of puppy	Penta-Ca phytate*	Ca phosphate	Vitamin D (i.u.)
33†	+	−	0
34	+	−	20
35	+	−	1000
36	−	+	1000

* Each batch was tested independently, the Ca:P ratios varying from 1·03 to 1·1.

† See note under Table 10.

Age at beginning of experiment: about 8 weeks; duration of experiment: 9 weeks.

It is seen from Text-fig. 12 that puppies 34 and 35, both of which had Ca phytate and vitamin D_2 in addition to the basal diet, absorbed sufficient Ca to maintain a positive balance of between 100 and 200 mg. daily throughout the experimental period, but that 35, receiving 1000 i.u. of the vitamin daily, absorbed no more than 34, which had only 20 i.u. Both, on the other hand, absorbed much less than puppy 36, which had the larger dosage of vitamin D_2 and the same amount of calcium as 34 and 35, but as phosphate instead of phytate. The calcium absorbed by the phytate animals during the experimental period could only have come from the Ca phytate in the food. The third of these, puppy 33, which had no vitamin D in its diet, lost its ability to absorb Ca after 6½ weeks of the experiment, in spite of the fact that it did not eat as much food as the others, grew less, and at an early stage of the experiment had one dose of 200 i.u. of vitamin D_2 injected into the blood stream (see note, p. 513), which no doubt lengthened the period during which absorption of Ca took place. It is therefore probable that in the complete absence of vitamin D the Ca of Ca phytate was unavailable to this dog.



Text-fig. 12 (Exp. 10). Relative effects of Ca phytate and Ca phosphate on Ca retention in the presence of different amounts of dietary vitamin D_2. About 85% of the Ca in the diet was given as Ca phytate in 33, 34, 35 and as Ca phosphate in 36. *Note.* In the absence of dietary vitamin D (33) the power to absorb Ca from Ca phytate was soon reduced. 20 i.u. of vitamin D_2 daily (34) promoted a higher retention of Ca, but 1000 i.u. (35) effected no further improvement. With equal amounts of vitamin D_2 (1000 i.u.), Ca phosphate (36) allowed a much higher Ca retention than did Ca phytate (35).

TABLE 10 (Exp. 10)

	Dietary conditions. Additions to basal diet									Bone results		
	Calcium				Phosphorus							
	Total*		Phytate		Total*		Phytate		Vitamin D_2 (i.u.)	Combined weight of ash of humerus radius and ulna	*A/R* ratio of femur shaft	Rickets† as judged by X-rays at P.M.
No. of puppy	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.				
33‡	212	366	191	322	319	469	200	300	0	3·077	0·958	5
34	272	366	256	322	408	469	250	300	20	4·656	1·103	0
35	272	366	256	322	408	469	250	300	1000	4·586	1·106	0
36	308	366	0	0	432	469	44	55	1000	5·129	1·312	0

* These figures were obtained from estimations on the cooked food. The Ca as phytate figures were calculated.

† Rickets graded 1–10, the number increasing with the severity of the disease.

‡ As this animal did not eat well, 200 i.u. of vitamin D_2 were injected intravenously on the 7th day. The appetite improved but it was not possible to give it the same amount of cereal and Ca phytate as the rest of the family until towards the end of the experiment.

When bone calcification is considered it will be seen that the mineral ash weights (Table 10), as would be expected from the Ca absorption, were low in 33, high in 36 and equal at an intermediate level in 34 and 35. The *A/R* ratios show that 36 (phosphate) had better calcified bone than the three phytate animals, but the differences between 33 (D-deficient), 34 (20 i.u.) and 35 (1000 i.u.) were small. This may have been due partly to the short experimental period and relatively poor bone growth and partly to the injection of 200 i.u. of vitamin D_2 given to 33.

Thus the results of this experiment are in keeping with the findings of Exps. 8 and 9, namely that phytate in the presence of vitamin D can reduce both the Ca absorption and the amount of Ca deposited in the bones to a greater extent than phosphate of equal P content, and further that a large increase in dietary vitamin D does not offset this effect. It also shows that an animal supplied with vitamin D cannot only make use of Ca given as an insoluble penta-calcium phytate but can in some way deal with the phytate and reduce the amount excreted. Thus when the diet contained vitamin D (20 or 1000 i.u. daily) only about 40–60 % of the P fed as phytate was recovered from the faeces.

The Interaction of Additional Calcium with Phytate in the Presence of Different Amounts of Dietary Vitamin D

It was shown in 1925 (Mellanby) that the addition of calcium to a low-calcium, vitamin D-deficient diet consisting largely of cereals resulted in improved calcification with a delay in the onset of rickets. In the following experiments an examination was made of the interaction of different amounts of dietary Ca with phytate as indicated by the absorption of Ca and phytate P and by calcification of bones.

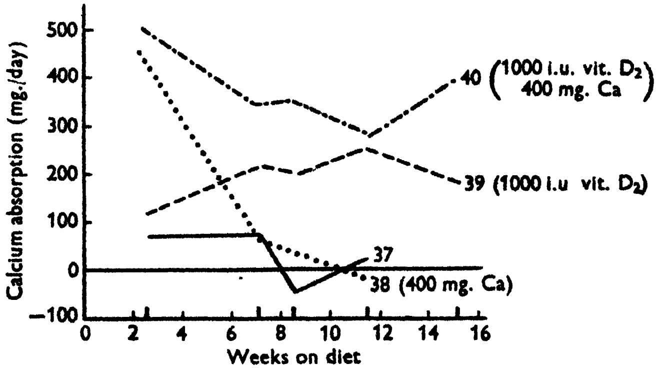
Exp. 11

Pre-experimental diet. **For about 2 weeks before the experiment was begun each of the puppies had 2·5 ml. of cod-liver oil, daily, so that all would have moderate bodily reserves of vitamin D.**

Basal experimental diet. **Bread, 64–150 g.; separated milk powder, 20 g.; lean meat, 7·5–17·5 g.; peanut oil, 5·6–13 ml.; NaCl, 0·75–1·75 g.; baker's yeast, 2·25–5·25 g.; ascorbic acid, 5 mg.; cabbage, 10·6–24·5 g.; Na phytate, 200 mg. (Ca content: initial, 290 mg.; final, 398 mg. P content: initial, 523 mg.; final 700 mg., phytate P content: initial, 216 mg.; final, 230 mg.)**

Age at beginning of experiment: 6½ weeks; duration of experiment: 15–16 weeks.

Text-fig. 13 shows that, in the presence of abundant dietary vitamin D_2, increasing the calcium intake led to a greatly increased calcium absorption, especially in the earlier days of the feeding period (see 40 as compared with 39).



Text-fig. 13 (Exp. 11). Effect of different dietary calcium intakes on Ca absorption in the presence and absence of vitamin D_2. *Note.* Ca absorption was highest throughout in 40 and 39 (1000 i.u. vitamin D_2) but was specially high in 40 (extra 400 mg. Ca). After 7 weeks of the D-deficient diet (37 and 38) the power to absorb Ca was much reduced even in the presence of an extra 400 mg. of Ca (38). 200 mg. P as sodium phytate were included in the daily diets of all.

When there was no vitamin D in the diet, but the reserves were moderately good, a high calcium intake, as in the case of puppy 38, also caused a large calcium absorption. As the reserves were used up, however, most of the additional dietary Ca was excreted and the amount absorbed diminished, so that by the 7th week of the experiment only about 50 mg. daily were absorbed as compared with 450 mg. in the early days of the feeding period. After this interval there was but little or no difference in the amount of calcium absorbed by puppies 37 and 38, receiving the lower and higher calcium diets respectively.

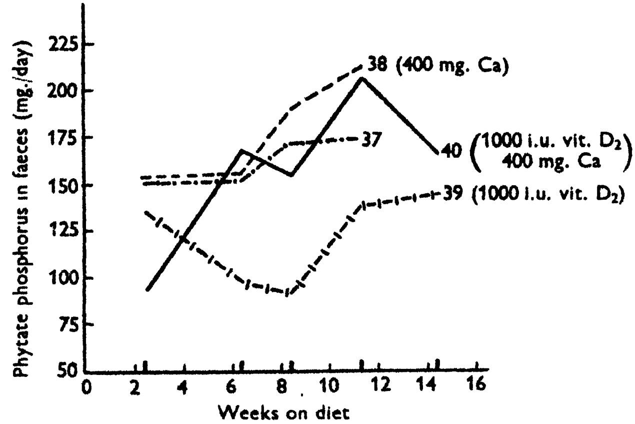
The effect of the large absorption of calcium by puppy 38 during the early weeks on the high Ca diet was greatly to delay the onset of rickets. After 11½ weeks of the experiment the radiographic appearance of this animal was nearly normal, whereas puppy 37, with no extra Ca, had developed very severe rickets and even at the end of the feeding period there was much less rickets in 38 than 37.

In view of the close relationship between phytate P and Ca excretion demonstrated in Exp. 8–10, it would be expected that the greatly increased excretion of Ca by puppy 38 following the loss of its vitamin D reserves would result in a corresponding excretion of phytate. Text-fig. 14 shows that this is not so, for, in the absence of vitamin D from the diet the additional Ca had but little effect on the phytate excretion. Puppy 38 (high Ca) even after 11½ weeks on diet excreted only about 38 mg. of phytate more than puppy 37 (low Ca). In the presence of dietary vitamin D_2 the addition of dietary Ca caused an increase in phytate excretion, the greatest variation between the puppy having additional Ca (40) and that not receiving it (39) being 77 mg. after 11½ weeks on diet. It will be seen that when no additional Ca was given, 40–60 % of the dietary phytate disappeared from the gut of the animal receiving vitamin D (39), whereas only 25–35 % disappeared in the case of the D-deficient animal (37). There

TABLE 11 (Exp. 11)

No. of puppy	Dietary conditions. Daily additions to basal diet: Ca as $CaCO_3$ (mg.)	Vitamin D_2 (i.u.)	Bone results: A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.*
37	0	0	0·43	10
38	400	0	1·19	2
39	0	1000	1·24	0
40	400	1000	1·53	0

* Rickets graded 1–10, the number increasing with the severity of the disease.
In this experiment 200 mg. of phytate P were included in the basal diet of all puppies.



Text-fig. 14 (Exp. 11). Effect of additional dietary Ca on phytate excretion in the presence and absence of dietary vitamin D_2. In the presence of vitamin D_2 (1000 i.u.), the addition of 400 mg. of Ca daily (40) increased the faecal phytate (cf. 39). In the absence of the vitamin, the additional 400 mg. Ca (38) did not materially increase the phytate excretion, since much of the phytate ingested was being excreted without the additional Ca (37).

was less margin, therefore, for the additional Ca to increase phytate excretion when the diet was deficient in vitamin D.

It is evident from this experiment that, even when there is no vitamin D in the diet, the body reserves of this substance can be very effective in promoting the absorption of any additional calcium there is in the food, but may have less effect on phytate excretion. The presence of dietary vitamin D promotes the absorption of some of the additional Ca, but that not absorbed appears to increase the excretion of phytate.

In the next experiment the effects of adding calcium to diets of animals containing smaller amounts of vitamin D_2 than were given in Exp. 10, namely 0, 5, 20 and 100 i.u. respectively, were tested as it was thought likely that the effect of raising the Ca intake might vary with the magnitude of the vitamin dosage.

Exp. 12

Pre-experimental diet. The litter, when fed independently of the mother, did not receive any additional source of vitamin D.

Basal experimental diet. Oatmeal,* 15–175 g.; separated milk powder, 25 g.; lean meat, 15 g.; peanut oil, 10 ml.; NaCl, 1 g.; baker's yeast, 5% of cereal; ascorbic acid, 5 mg.; vitamin A acetate, 1500 i.u. (Ca content: initial, 340 mg.; final 443 mg. P content: initial, 326 mg.; final, 945 mg., phytate P content: initial, 38 mg.; final, 438 mg.)

Age at beginning of experiment: 6 weeks; duration of experiment: 17 weeks.

It will be seen from Table 12 and Text-fig. 15 that:

(1) Raising the Ca intake by the addition of $CaCO_3$ increased the amount of Ca absorbed both in the absence and in the presence of vitamin D, but in the former case the increase was relatively small.

(2) The effect of added Ca varied with the amount of vitamin D given. Thus a dose of 5 i.u. of vitamin D_2 with additional Ca (44) resulted in approximately the same Ca absorption as 100 i.u. without the additional Ca (47).

(3) Increasing the vitamin D intake from 0 to 100 i.u. when the Ca of the food was low, increased the A/R ratios of the bones (0·85, 1·04, 1·11 and 1·27) but when the Ca content of the diet was higher the only increase in vitamin D intake which produced any significant improvement in calcification was that from 0 to 5 i.u. daily.

This experiment indicates, therefore, that under these conditions the addition of $CaCO_3$ to the diet promotes the absorption of Ca, and that the extra Ca content is of greater significance to the animal's economy when the dietary vitamin D is small.

As regards bone calcification, animals 41 and 42 show that an increased Ca absorption in the absence of dietary vitamin D does not necessarily indicate improved bone quality. Thus puppy 42 (Text-fig. 15*b*) consistently absorbed more

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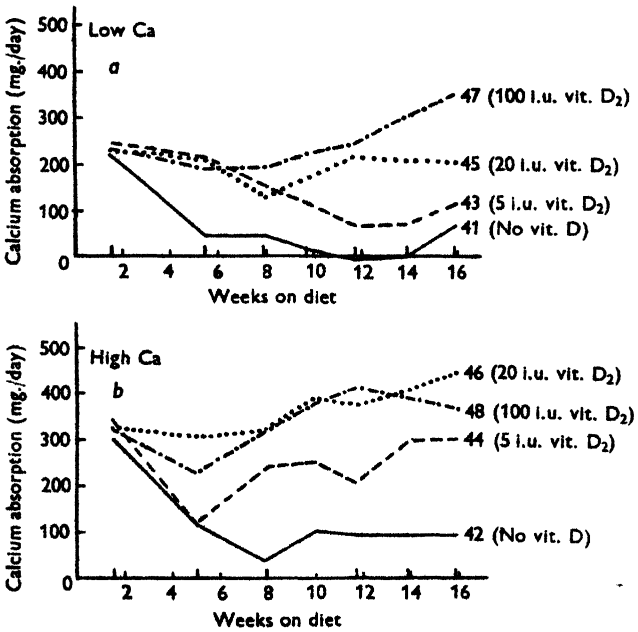
* For a family of this size it was considered better to use oatmeal as a source of phytate than to attempt the preparation of large quantities of Na phytate.

TABLE 12 (Exp. 12)

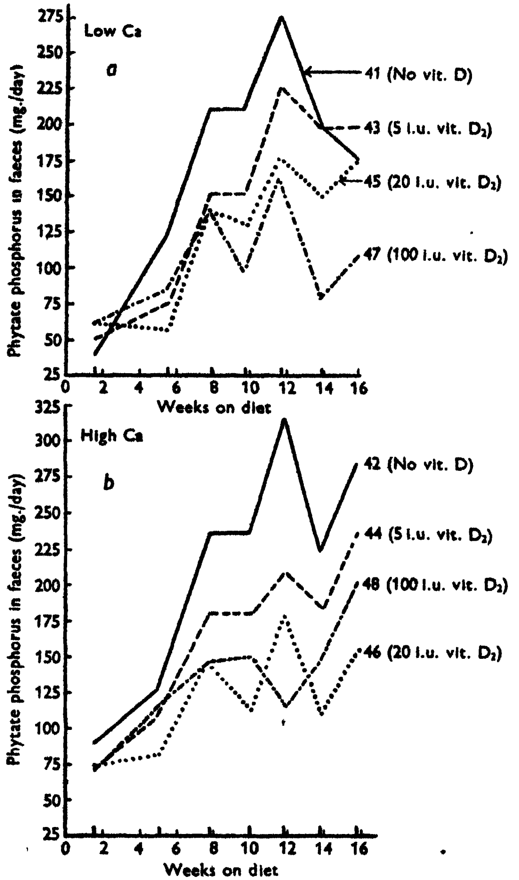
	Dietary conditions. Daily additions to basal diet		Bone results	
No. of puppy	Calcium as $CaCO_3$ (mg.)	Vitamin D_2 (i.u.)	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.*
41	0	0	0·85	9
42	200	0	0·88	8
43	0	5	1·04	3
44	200	5	1·30	1
45	0	20	1·11	1
46	200	20	1·35	0
47	0	100	1·27	0
48	200	100	1·36	0

* Rickets graded 1–10, the number increasing with the severity of the disease.



Text-fig. 15 (Exp. 12). Effect of additional dietary Ca on Ca absorption in the presence and absence of vitamin D_2. (a) With low Ca intake increasing vitamin D causes increased calcium absorption; (b) with a higher Ca intake increased vitamin D increases the absorption of Ca except that 46 (20 i.u.) and 48 (100 i.u.) are practically the same (cf. phytate excretion Text-fig. 16b). *Note.* Comparison of (a) and (b) shows (1) that at all vitamin levels extra dietary Ca has caused increased absorption; (2) that 20 i.u. of vitamin D_2 with an additional 200 mg. of Ca (46) had a better effect on Ca absorption than 100 i.u. without extra Ca (47). Similarly, 5 i.u. vitamin D_2 with 200 mg. extra Ca (44) brought about better Ca absorption than 20 i.u. vitamin D without extra Ca (45).

Ca than 41. The mineral ash content of the femur shaft was 23 % greater than that of 41, but the *A*/*R* ratios (0·88 and 0·85) did not differ significantly and both animals had severe rickets. The converse of this, that equal ash content does not imply equal bone quality, was shown in Exp. 9.



Text-fig. 16 (Exp. 12). Effect of additional dietary Ca on phytate excretion in the presence and absence of dietary vitamin D. (*a*) With low Ca intake increasing vitamin D_3 causes a reduced excretion of phytate; (*b*) with a higher Ca intake increasing vitamin D reduces phytate excretion except that 46 (20 i.u.) and 48 (100 i.u.) are practically the same. *Note.* Comparison of (*a*) and (*b*) shows that (1) in the absence of vitamin D, additional Ca raised the faecal phytate; (2) when vitamin D was given in doses of 5, 20 and 100 i.u. respectively the effect of additional Ca on phytate excretion was small or absent.

Turning now to phytate excretion, Text-fig. 16*a* shows that with the lower Ca intake the phytate excretion was reduced as the dietary supply of vitamin D increased. In the presence of the additional Ca (Text-fig. 16*b*) diets containing 5 and 20 i.u. promoted progressively lower phytate excretions but increases beyond 20 i.u. had apparently no further action. Comparing the effect of the additional Ca in animals with otherwise identical diets, it will be seen that in the absence of the vitamin the excretion of phytate was slightly increased, but as the dosage of the vitamin was raised from 5 to 100 i.u. daily the slight effect of the additional Ca disappeared.

The reduced effect of the additional Ca on phytate excretion in the presence of a supply of vitamin D may have been due to the fact that most of the additional Ca was absorbed, so that the amount available for precipitating phytate was similar in each pair of animals having the same amount of dietary vitamin D. Had larger quantities of Ca been added to the diet, a stage would undoubtedly have been reached when the faecal phytate would have been greatly increased.

DISCUSSION

Experiments have been described above which formed part of a further study of the anti-calcifying effect of diets containing phytate as compared with those containing an equal amount of P in the form of phosphate. The immediate problem was to see whether this action of phytate was exerted in the presence of vitamin D.

When it was first established that phytate was the substance in oatmeal and other cereals mainly responsible for their anti-calcifying action, it was thought to be of but little practical importance so long as there was plenty of vitamin D in the food. The results described above showed that this was not the case. In the presence of vitamin D either in the food or when stored in the body, phytate consistently reduced Ca absorption to a greater extent than an equal amount of P given as phosphate. It is true that vitamin D, above a certain minimal level, prevented the development of rickets and of osteomalacia when the diet was rich in phytate and low in Ca, but even when the vitamin D intake was as high as 1000 i.u. daily, phytate still retained the power of reducing the absorption of calcium, thereby tending to produce a more subnormal condition of bone calcification than would be produced by a similar diet containing the same amount of phosphorus but as phosphate.

When it became apparent that the presence or absence of vitamin D was a crucial condition of the problem under study, one of the methods used in the foregoing experiments was to give the puppies a diet which caused the gradual exhaustion of their body stores of the vitamin. A great reduction in the power to absorb Ca was regarded as coincident with the exhaustion of these reserves. The period necessary to produce this condition varied greatly according to the quantity of the vitamin consumed and stored before the experiment began. In some cases it took 2 or 3 weeks only, whereas in others it might take two or more months on a vitamin D-deficient diet before the growing puppy lost its power to retain Ca. In adult dogs where the demand was probably less and the stores greater it might take a year before the body was deprived of all traces of the vitamin. In the early stages of the experimental feeding period before vitamin D depletion, phytate significantly lowered Ca absorption when compared with phosphate, but later this differentiation disappeared and the absorption was reduced in both cases, leading, if the experiment continued long enough, to negative balances of this element. It was not the effect of phytate in depressing Ca absorption which was fundamentally altered by the loss of vitamin so much as that inorganic phosphate changed from being relatively without effect on Ca absorption to having an inhibitory action of the same order as phytate.

In the foregoing experimental work two methods of assessing Ca metabolism have been used, (i) the determination of Ca and P absorbed and excreted by the gut under controlled dietary conditions, and (ii) estimation of the degree of calcification of the bones. Although it is generally true that more or less Ca absorbed from the intestine means better or worse calcified bones, the two measurements are not identical in their interpretation. Increasing the vitamin D supply of the body from nil upwards improves Ca absorption greatly once the stores of vitamin D are depleted, but soon reaches a point beyond which further increases of the vitamin have no such effect. It was found, for instance, that 20 i.u. daily of vitamin D_2, which will be referred to as the 'limiting amount', were often just sufficient with the experimental diets used to promote the maximum amount of Ca absorption, and that raising the dosage from 20 to 1000 i.u. in diets in which all other factors were constant did not materially increase the Ca absorption or retention.

Turning now to the question of the structure and calcification of bone, it would be expected that below the limiting amount of vitamin D, bone calcification would be poor and that rickets would be produced, since the amount of Ca absorbed was subnormal. Above this limiting intake, however, since the amount of Ca absorbed remained a maximum under those experimental conditions, it would be expected that the Ca content of comparable bones would also be a maximum, i.e. as much in amount in animals with low as in animals with higher vitamin D intake. This, indeed, proved to be the case but it did not mean that bones of animals receiving the larger amount of vitamin D were of the same structure, even though the Ca content was similar. In such cases the radiographic appearance and the A/R ratio of comparable bones sometimes showed that the amount of vitamin D which just promoted a maximum absorption of Ca was not sufficient to produce well calcified bones.

This fact can be seen in Pl. 2 (*e*, *f* and *g*) which shows radiographs of the costochondral junctions of three animals, two of which (*f* and *g*) had received a high phytate diet together with 20 and 1000 i.u. respectively of vitamin D. It will be seen that in puppy 28 (*f*) the ribs are thick and osteoporotic, whereas in puppy 32 (*g*), with the higher vitamin intake, they are relatively well calcified and well formed, yet the bones of these two animals contained approximately the same amount of mineral matter. In spite of this the A/R ratios of the femur shafts were in line with the radiographic appearance of the ribs, being less in the animal with the lower vitamin D intake (Table 9).

Since the A/R ratio represents the proportion of the total mineral content of the bone to the dried fat-free organic matter, the bone with the lower A/R ratio, but with the same total amount of mineral matter, must have had a larger quantity of non-calcified or poorly calcified organic matter, a fact confirmed by radiological and histological examination. The bones of puppy 28 were more cancellous, i.e. they contained more marrow spaces and were also of a more elementary lamellar type of structure with fewer well-formed Haversian systems than those of puppy 32 (1000 i.u. vitamin D_2). Of course with a vitamin D intake below the limiting amount, under the experimental conditions chosen, the Ca absorption from the gut was depressed and frank rickets with a large amount of osteoid tissue was produced. In this case the A/R ratio diminished, both because the mineral content was low and because non-calcified tissue, including osteoid tissue, was relatively increased.

Here, then, is direct evidence that vitamin D not only has the function of controlling Ca absorption and of incorporating it into bone, but above the limiting amount this vitamin controls the quality and structure of bone laid down, i.e. ensures that not only the pattern of the bone is correct but also that what is formed is more fully calcified, by limiting the formation of some non-calcified or partially calcified organic matrix which may not be osteoid tissue. The amount of vitamin D necessary to produce perfect bones is therefore, under the experimental conditions studied, higher than the amount necessary to produce maximum Ca absorption from the gut and maximum Ca incorporation in the bone.

Effect of phytate

The effect of increasing dietary phytate in the presence of vitamin D is quite another matter. In numerous experiments described above it has been shown that substituting sodium phytate for phosphate, so as to retain the same total P in the diet, has two constant effects. In the first place it increases the phytate in the faeces and the more consumed the greater is the amount excreted in this way (Text-fig. 9*a*). It is true that a varying proportion of the phytate eaten disappears, i.e. does not appear in the faeces or urine, and that the amount which disappears is greater when the diet contains vitamin D than when the vitamin is absent from the body. But this action of vitamin D in bringing about the 'disappearance' of phytate from the gut is limited so that, even when the vitamin D intake is raised to a higher level, other conditions being constant, there is no further diminution in the amount of phytate excreted. In other words, the limitation, referred to above, of vitamin D in bringing about Ca absorption, is also accompanied by a similar limitation in causing the disappearance of phytate from the intestine.

The second effect of adding phytate to the diet is to increase the Ca excretion in the faeces. Thus an increase in faecal phytate is associated with an increase in faecal Ca (Text-fig. 9*b*), which means that less Ca is available for absorption. In growing animals this may result in the production of rachitic or osteoporotic bones according to the intensity of the effect and the state of the animal at the time. If the vitamin D intake is just sufficient with the basal diet used to prevent rickets, then the addition of phytate, even if it replaces phosphate, will result in frank rickets. With higher vitamin D supplies, instead of producing rickets, the addition of phytate will tend to produce a more osteoporotic condition of the bone. This action of phytate on bone structure can be seen in Pl. 2 (*e*, *f* and *g*), showing radiographs of the costochondral junctions of three puppies. The ribs of (*f*) are thick and osteoporotic while those of (*g*) are thinner and more normal. Both these animals received 20 i.u. vitamin D_2 daily and the osteoporotic appearance of (*f*) is due to the larger calcium-depriving effect of phytate as compared with that of phosphate (*e*).

Under some conditions phytate may even cause withdrawal of Ca from the body itself. This was shown experimentally in its grossest form by maintaining fully grown dogs on diets poor in Ca and vitamin D and rich in high phytate-containing foods such as oatmeal and maize (Mellanby, 1937). These animals developed severe osteomalacia and osteoporosis with great bone deformity. Additional vitamin D protected animals on these diets to some extent, while vitamin D and a sufficiency of Ca gave full protection. Animals on similar diets in which rice or white flour (poor in phytate) replaced the oatmeal and maize, did not develop these gross deformities although the bones were osteoporotic. Thus it will be seen that dietary phytate has the power not only of immobilizing Ca in food but may even be responsible for its withdrawal from the highly calcified tissues of the body such as bones and for causing its loss through the intestinal tract.

The importance of a high calcium intake with phytate

The practical issue, therefore, is how best to bring about perfect bone formation in growing animals when the diet is relatively rich in phytate, as, for instance, when it contains abundant cereals such as oatmeal or maize. The objective must be to increase the Ca absorption so that it can be incorporated to the best advantage in the bone and other tissues. Obviously a sufficiency of vitamin D is essential, but it is just as important at this stage to increase the Ca intake. There are two reasons for this. In the first place, under the experimental conditions chosen, calcium is often a limiting factor in the diet. Secondly, increasing the Ca makes the vitamin, especially if present in small quantities, much more effective. For instance, a puppy which received 5 i.u. of vitamin D_2 and an additional 200 mg. daily of Ca (Exp. 12) absorbed a quantity of Ca approximately equal to that absorbed by another puppy of the same litter getting 100 i.u. of the vitamin without the extra Ca (puppy 47). This synergistic effect of Ca salts with the anti-rachitic vitamin has long been known and was described in 1925 when it was found that the calcium retention produced by butter was greatly increased if additional Ca carbonate or Ca phosphate was present in the food (Mellanby). It was this fact which formed the basis for emphasizing at that time the advantages of giving milk rather than an equivalent amount of butter for the calcification of bones in children.

It might be thought that increasing the calcium would only bring about the formation of more insoluble pentacalcium phytate in the intestine and thereby increase both the phytate and the Ca excretion, with no benefit to the animal. It is, of course, true that if the calcium added is excessive and the P intake low such an effect will be produced, but increasing the Ca intake within physiological limits, although causing a slight increase in phytate excretion, also increases the Ca absorption. The idea that the addition of calcium to the diet causes a great increase in the phytate excretion is probably dependent upon rat experiments, a point which will be referred to in more detail later. So far, however, as the dog experiments are concerned, it is undoubted that one way, and the most important practical way, of overcoming the Ca immobilizing effect of a diet rich in phytate, assuming that there is some vitamin D present in the body or diet, is by increasing the calcium intake. A diet which contains vitamin D and is rich both in calcium and phytate is compatible with perfect bone formation, but this is not the case when the diet is rich in both vitamin D and phytate and relatively (but not absolutely) low in calcium. Increasing the Ca intake when the body and the diet are devoid of vitamin D is of little use since much, or in some cases all, of the extra Ca is excreted, together with most of the phytate.

The main fact to be gathered from these experiments is that, once sufficient vitamin D is given, the level of Ca absorption is controlled by the amount of Ca and by the amount and proportion of dietary P given as phytate.

Inconsistency of dog and rat experiments as regards phytate effect

It may be asked why it has taken so many years for the anti-calcifying action of cereals to be generally accepted. Much of the criticism which has been directed against the work has depended on results showing that rickets in rats is controlled by the Ca:P ratio of the diet and the availability of P. Can the present results explain the apparent anomalies between rat and dog investigations? In order to produce rickets in rats with certainty it has been usual to deprive the mother of vitamin D during lactation, give a vitamin-D free diet to the young and use an experimental diet having a high Ca:P ratio. This unnatural dietetic technique and its results have largely dominated the subject of the human disease.

The effect of the complete deprival of vitamin D would certainly tend to increase the significance of the Ca:P ratio of the diet in the aetiology of rickets for, as was demonstrated in the above dog experiments, phosphate becomes under such conditions nearly as anti-calcifying, in the sense of preventing Ca absorption, as phytate. The second condition of the rat experiments, namely the high Ca-low P diet, must also have a special but abnormal significance. Clearly with diets containing four or more parts of Ca to one of P, the factor most powerfully determining the degree of calcification is the availability of the P, for however much Ca is absorbed it cannot be incorporated into growing bone unless there is a sufficiency of P with which to combine. The general effect of adding very large amounts of Ca to the diet is to immobilize by precipitation both the phytate and phosphate P of the diet and prevent its absorption. The significance of P as the limiting factor in experiments on rickets was particularly apparent when Bruce & Callow (1934) showed that with diets of high Ca content phytate in the presence of vitamin D was less effective than phosphate in promoting healing of rickets. The problem of the relative availability of phytate and phosphate in rats was further studied by Krieger & Steenbock (1940), both in the presence and absence of vitamin D, with diets the Ca:P ratio of which varied over a wide range. As a measure of availability of the P of phosphate and phytate they estimated the degree of calcification of the bones. They found that, in the absence of vitamin D and with an optimal intake of P and a Ca:P ratio of 1:1 the utilization of phytate P and phosphate P was not greatly dissimilar. With higher Ca:P ratios of 2:1, 4:1 and 6:1 the availability of the phytate P decreased rapidly, whereas the inorganic phosphate remained available to the optimum extent. When, on the other hand, there was vitamin D in the diet, the effect of altering the Ca:P ratios was not so apparent, for the vitamin improved the utilization of both phytate and phosphate P at all Ca:P ratios. Nevertheless, even in the presence of vitamin D, phytate P was never so readily available as the inorganic form.

These experiments show that phytate and phosphate have different biological availabilities in rats irrespective of the presence or absence of vitamin D, and that these differences only become evident when the P supply to the growing animals is limited. They do not throw any light, however, on the main point at issue, namely whether phytate in the diet is more anti-calcifying than phosphate, i.e. more potent in preventing Ca absorption and utilization, especially under what might be regarded as normal dietetic conditions, when, for example, the Ca:P ratio is less than or approaching 1, and there is at least some reserve of vitamin D available. Green & Mellanby (1928) investigated the effect of such diets on rats and their results indicated that phytate (although not recognized at that time as the offending agent) had a more powerful anti-calcifying effect than phosphate. They showed among other things that oatmeal (high phytate) was more rachitic than white flour (low phytate). Unfortunately it has proved impossible to confirm these earlier results on rats with any consistency and it must be assumed that some unrecognized condition was present in that work. Many experiments were carried out at that time and the results obtained were so consistent that it is impossible to put them aside as fortuitous. The later discovery of Patwardhan (1937) that the mucous membrane of the intestine of rats is rich in phytase suggests that these animals have a much greater power of hydrolysing phytate to inositol and phosphoric acid than other experimental animals tested and that it is this factor which explains the greater difficulty of depressing bone calcification by phytate in rats. The fact that in rats phytate P only becomes much more difficult to absorb than phosphate in the presence of a very high Ca intake supports this view. Possibly in the type of rat used in the work of Green & Mellanby or under the conditions of their experiments the phytase activity of the gut was abnormally small. However that may be, the evidence indicates that the anti-calcifying effect of phytate is much smaller in the rat than in the dog, and that this difference is probably due to the greater phytase content of the rat intestine.

It is clear, therefore, that if the interpretation of the aetiology of rickets as it occurs in human beings had to depend on past experimental work on rats, it would be a right assumption that the Ca:P ratio and the availability of P of the diet explained the action of cereals in this disease. There would indeed be little or no support for the view that cereals differed in their rickets-producing action and that this was dependent largely on their content of phytate. On the other hand, the present experiments have abundantly confirmed those of Harrison & Mellanby and shown that in dogs phytate as compared with phosphate has the specific property of reducing the absorption of Ca, especially in the presence of vitamin D. In the dog experiments the Ca:P ratio has proved to be but a poor indication of the relation of diet to rickets. The vitamin D content of the body is always dominant but the intensity of its action as regards calcification can be modified in one direction by adding Ca and in the other by adding phytate. It has been shown above that diets having the same Ca:P ratios and amounts may have different effects on Ca absorption and bone calcification according to the chemical nature of the P-containing substance; again, altering the ratio by increasing the phosphates of the diet often has but little effect on Ca absorption. Indeed, it can be said that the amounts of dietary Ca and P necessary to produce optimal bone calcification vary greatly according to the other constituents of the diet and cannot be regarded as having any constant significance. It will be seen that the basis of the ordinary technique used to produce rickets in rats, namely severe limitation of P and excess of Ca intake, has only a partial relation to the nutritional condition which produces the human or canine disease where the main determining factor is the absorption of Ca in a sufficient and a correct form and not a deficiency either in the supply or absorption of P. The rat, as an experimental animal in the elucidation of the aetiology of rickets as it occurs in human beings, has in some respects proved a defective guide and has long prevented the acceptance of the fact that some cereals produce defective calcification and that this action is dependent on the phytate content and Ca limitation.

The toxamin or anti-vitamin action of phytate—phytate and phosphate as chemical analogues

It may be of interest to discuss briefly the anti-calcifying action of phytate in the light of other recent discoveries on the antagonistic action of structural analogues, which have not only greatly illuminated the subject of anti-vitamins in general but have opened up many other biochemical problems, including the important field of chemotherapy. It was stated in the introduction that when the anti-calcifying action of cereals, and especially of oatmeal, was first reported, the hypothetical substance responsible for this action was described as an anti-vitamin, but that later the word 'toxamin' was used as being more descriptive of its effects. As the chemical nature of the cereal anti-vitamin was not known at that time no suggestions as to the basis of the antagonism were possible. The word 'toxamin' was substituted for anti-vitamin in this instance in order to describe a substance present in some cereals which, by interfering with Ca metabolism, had a harmful effect on the body. It was thought at that time that vitamin D could effectively prevent this action. The present work shows that this is not entirely the case but that, although vitamin D can usually cloak the harmful action of phytate, especially as regards the production of florid rickets, it cannot by itself prevent some anti-calcifying effect. Kodicek (1948) has suggested that the term 'toxamin' should be applied to all substances which interfere with the action of vitamins and that the term 'anti-vitamin' should be reserved for substances which act by reason of chemical analogy. Phytate of cereals, under this definition, qualifies for the designation 'toxamin' because it interferes with the action of vitamin D, probably not by direct antagonism to the vitamin, but indirectly by limiting the amount of Ca available upon which the vitamin can work. It is recognized, however, that this explanation may not account for all the actions of phytate in relation to vitamin D.

There are obviously a number of ways in which a vitamin can be prevented from doing its work. It may be destroyed in the intestine, or if present in the food or formed in the gut by micro-organisms, it may not be absorbed for some reason, but these instances are not due to the presence of an anti-vitamin. Anti-vitamin action may depend on the presence of a substance in food which is a structural analogue of the vitamin and displaces it from the surface on which it catalyses certain essential chemical reactions. In such an instance, while the anti-vitamin has a structural resemblance to the vitamin, it also has a structural difference which prevents it from having the biological action of the natural vitamin. Although this is a widely accepted hypothesis, advanced to explain certain cases of biochemical interference of this type, not only in the case of some vitamins but of other substances having a drug-like action, there are certainly other possible modes of interfering action of structural analogues. For instance, an anti-vitamin may not interfere directly with the vitamin but may act by removing the substrate from its sphere of influence. This latter type of action is more likely to be the main explanation of the present problem of interference of phytate in calcification. As regards Ca absorption from the gut, the antagonism between phytate and vitamin D does not seem to be direct, otherwise a more evident quantitative relationship would exist between the two substances. As has been shown in Exp. 8 above, however, increasing the vitamin D of the diet from 20 to 1000 i.u. did not further antagonize the power of phytate to reduce Ca absorption from the gut. On the other hand, as also shown above, there was good evidence of a direct antagonism between phytate and phosphate in regard to Ca.

In what form the calcium passes from the gut into the blood stream is not known, but it may be that its absorption involves a reaction of a complex containing Ca and phosphate in a form related to the compound which is ultimately deposited in bones. In the complete absence of vitamin D from the body, whereas little Ca is absorbed, the absorption of P may be relatively large. On the addition of the vitamin to the food the absorption of Ca is immediately resumed and with this the P absorbed is also increased. The assumption therefore is that, under the influence of vitamin D, Ca is absorbed in association with phosphate. The view that the absorption of Ca is the dominant factor in the action of vitamin D on the intestine is in agreement with that of Nicolaysen (1937). He demonstrated that, in rats on a low P diet, Ca absorption was greatly reduced in vitamin D deficiency, but that when dietary Ca was low and the P moderate the latter was absorbed to an equal extent both in the presence and absence of the vitamin. He suggested that the reduced absorption of P reported in vitamin D-deficient rats was due to the large amount of unabsorbed Ca in the bowel precipitating the P. It may not be out of place to remark here again that the unphysiological high Ca-low P diets used to produce rickets in rats may be misleading, for in dogs with diets of more natural Ca:P ratios it appears that the reduced P absorption in vitamin D deficiency is due to the fact that when the Ca is not absorbed less P is needed and not, as in rats, to the precipitation of the phytate P by the large amount of unabsorbed Ca in the gut. Assuming, therefore, that Ca is absorbed in some combination containing phosphate, it follows that phytate, by combining with Ca to form the insoluble, quickly precipitated penta-calcium phytate (Hoff-Jørgensen, 1944), effectively reduces the formation of any complex containing the more soluble and more slowly precipitated calcium phosphate and thereby the Ca available for absorption. Here, then, is an instance of structural analogues, phytate and phosphate, competing for a third substance, Ca, the absorption of which is controlled by a vitamin.

Whereas this part of the antagonism between phytate and phosphate and vitamin D is reasonably clear, it is more difficult to understand how vitamin D prevents the phytate effect becoming complete master of the situation. It does this at least partly by establishing conditions favourable to the breakdown of a large part of the phytate in the diet. How powerful this action can be is seen in Exp. 10 above, where it was shown that, even when the sole source of calcium in the diet was in the form of Ca phytate, the body absorbed a fair amount of calcium when there was a source of vitamin D in the body or in the food. It is true that the amount absorbed was much less than when the dietetic Ca was in the form of calcium phosphate; for instance, in Exp. 10, 218–348 mg. of Ca were absorbed daily in the phosphate animals when practically the only source of Ca was calcium-monophosphate, whereas in the corresponding phytate animal only 85–199 mg. of Ca were absorbed. It is difficult to believe that so much of the Ca of the phytate compound would have been absorbed if some of the phytate had not been converted to phosphate, and there is evidence that this actually happens under these conditions. In Exp. 10 it was found that 40–60 % of the phytate consumed disappeared from the gut. This disappearance of the phytate caused by vitamin D will be described and discussed in a later publication.

In the complete absence of vitamin D, both from the diet and from the body, very little Ca is absorbed from the alimentary canal. As the vitamin D content of the diet increases, the amount of Ca absorbed increases, but now the antagonism between phytate and phosphate comes into play, the amount of Ca absorbed being lower in animals receiving dietary phytate. Vitamin D, probably by promoting the hydrolysis of phytate to phosphate, reduces this effect, but even a large addition of the vitamin does not permit destruction of all the phytate and the Ca absorption remains subnormal. Apart from its action in the gut, phytate appears to have little, if any effect, and vitamin D takes full control, improving the calcification of bones both by depositing a Ca-phosphate complex in osteoid tissue and by reducing the proportion of other organic as compared with inorganic material. Even with large supplies of vitamin D, however, unless additional Ca is given to satisfy the undestroyed phytate in the intestine, the calcification of the bones of animals receiving large amounts of dietary phytate will remain subnormal as compared with that of animals whose phosphorus is given as phosphate.

There is still one further aspect of phytate and vitamin D relationship which, while obviously a part of the general problem, evades explanation. This is a point which was indicated in Exp. 7, namely that dietary phytate seems to impose an increased demand on vitamin D. In that experiment it appeared that phytate hastened the disappearance of body reserves of this vitamin.

Inorganic phosphate may have a similar action, but, if so, it is of a lower intensity than that of phytate. The explanation of an antagonism of this kind, if confirmed by further work, would require more than the Ca-immobilizing effect of phytate, which seems to be the main explanation of the latter's action. The interplay of vitamin D, Ca, inorganic phosphate and phytate is still far from being understood.

Phytate and phosphate in human dietary

Finally it may be asked whether the factors described above apply to human beings. There is good evidence that they do. For instance, McCance & Widdowson (1942*a*, *b*) have shown: (1) that Ca was much less freely absorbed from diets consisting largely of brown bread than from those consisting largely of white; (2) the amount of Ca absorbed from brown bread diets could be raised by adding Ca to the diet; (3) the absorption of Ca from white bread could be prevented by adding sodium phytate to it; (4) the absorption of Ca from brown bread diets could be improved by dephytinizing the bread, i.e. by allowing the phytase of the flour to hydrolyse the phytate to inorganic phosphate.

Danish workers have also studied the problem and found that phytate interferes with the absorption of Ca in infants (Hoff-Jørgensen, Andersen, Begtrup & Nielsen, 1946) and in children (Hoff-Jørgensen, Andersen & Nielsen, 1946). So far as the general problem is concerned, namely the specific action of phytate in lowering the absorption of Ca from the gut, human beings undoubtedly react in the same way as dogs but probably unlike rats. The partial destruction of phytate also appears to take place in the human alimentary canal as in dogs and, according to Cruickshank, Duckworth, Kosterlitz & Warnock (1945) the amounts so destroyed may be large when the diets are sufficiently low in Ca to give a negative balance. The same workers showed that an addition of Ca to these diets diminished the phytate destruction if the Ca and the source of phytate were eaten at the same time. If there was an interval between the consumption of these two food factors, for instance, if the phytate as a component of oatmeal was given at breakfast and the addition of Ca in the form of milk at supper, there was no diminution in the amount of phytate which disappeared from the gut.

Some workers, including McCance & Widdowson (1942*a*) and Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946), when testing the action of vitamin D on Ca absorption in human beings, have obtained negative results. Such results can well be understood in the light of the foregoing dog experiments, for the amount of vitamin D necessary to promote a maximum Ca absorption is small and can be long supplied from the body reserves. Its administration might, therefore, result in little or no increase in Ca absorption, and might indeed have no effect which could be detected on the basis of our present knowledge, unless the body had previously been depleted of its stores. This long-continued effect of body stores of vitamin D in man was evident in the work of Hannon, Liu, Chu, Wang, Chen & Chou (1934), who found that a small daily dose of vitamin D (amount unstated) given to adult osteomalacic patients over a period of 16 days, retained its influence on Ca absorption for at least 4 months after the administration had ceased. It is also well recognized that the assay of vitamin D can only be made on young animals after they have been depleted of their reserves of this substance. It now appears that in adults also the effect of vitamin D on Ca absorption only becomes evident when the body is depleted of or deficient in this vitamin.

Recently Walker, Fox & Irvine (1948), in confirming the reduction in available Ca that is produced in man by an increase in dietary phytate (as brown bread), have concluded that adults can accustom themselves to a higher phytate intake so that in time (about 6 weeks) a negative Ca balance is changed to a positive one. No good evidence of this adaptation has been seen in the present experiments on puppies, nor would it have been expected because the phytate content of the diet increased gradually during the experimental period.

While it is undoubtedly true that the body has some power of accommodating itself to changes in Ca intake, as indeed it has to many other environmental changes, it remains to be shown what the long-term effect of high phytate diets would be, and whether, assuming Ca equilibrium were maintained, the body reserves of Ca would be kept at a level consistent with optimal physical fitness. In this connexion it may be mentioned that Henderson & Kelly (1929) reported that a Ca balance could be maintained in growing boys at a daily intake of 430 mg. On giving additional Ca in a mineral mixture it was found that after the maintenance requirement had been satisfied, as much as 50% of any excess might be retained. The Ca of milk, on the other hand, was found to be utilized more efficiently, the corresponding retention figure being over 70%.

Meanwhile, it would be unfortunate if emphasis on such adaptations as may occur in adults were to obscure the major importance of a physiological sufficiency of available Ca, especially in children and growing animals. The practical objective, therefore, ought to be, not the Ca intake which just prevents a negative balance, but the Ca intake which promotes maximum absorption and retention, assimilated with sufficient vitamin D to ensure the optimum incorporation of the Ca in perfectly formed bones and teeth.

SUMMARY

1. A study has been made of the conditions affecting the action of cereal phytate in interfering with calcium metabolism and calcification processes in the growing animal (dog). The anti-calcifying effect of phytate is specific when the body contains vitamin D. In the complete absence of the vitamin from the diet and the body the specificity of action is largely lost and the anti-calcifying action of inorganic phosphate approaches that of phytate.

2. The anti-calcifying property of cereals was formerly ascribed to an anti-vitamin and later to a toxamin, a substance having a harmful effect which could be antagonized by the vitamin. Phytate does not seem to be an anti-vitamin in the sense that it directly antagonizes vitamin D, although this hypothesis may be necessary to explain some of its less understood interactions with the vitamin (see paragraph 9 below). Its main anti-calcifying effect depends on the fact that it competes in the intestine with its structural analogue, inorganic phosphate, for Ca. In this way phytate limits the amount of Ca available for absorption under the influence of vitamin D.

3. Although vitamin D in sufficient quantity prevents phytate from producing rickets in growing animals, it alone does not suppress the anti-calcifying effect of phytate. However high the vitamin D intake, phytate reduces the amount of Ca absorbed from the intestine and produces bones less well calcified and more osteoporotic than when it is absent from the diet or replaced by inorganic phosphate.

4. Increasing the phytate in the diet increases not only the phytate but also the Ca in the faeces, and in this way reduces the Ca available to the body.

5. A high phytate diet demands not only an adequate vitamin D supply, but, equally important, a high Ca intake. When the diet is rich in phytate, perfect bone formation can only be procured if sufficient Ca is added to a diet containing vitamin D.

6. Increasing the Ca of the diet may: (*a*) increase the excreted phytate if the absorption of Ca is not increased; or (*b*) reduce the phytate in the faeces if more Ca is absorbed.

7. Under the experimental conditions described, vitamin D does not remain passive to the antagonism of phytate but reacts by destroying some of it. This action is limited, and increasing the vitamin above a certain amount does not further reduce the phytate excreted, just as it does not increase the Ca absorbed.

8. Vitamin D not only promotes Ca absorption from the gut and its deposition in bone, but in growing animals adjusts the structure and bulk so as to produce better calcified bones in that they have a maximum of calcified tissue. More of this vitamin may be required to ensure perfect bone calcification than to procure maximum Ca absorption, and this is specially so when the diet is rich in phytate. Thus Ca absorption and retention may be good and yet the calcification of the bones be subnormal.

9. There is some evidence, not fully substantiated, that dietary phytate uses up the body reserves of vitamin D more rapidly than does inorganic phosphate.

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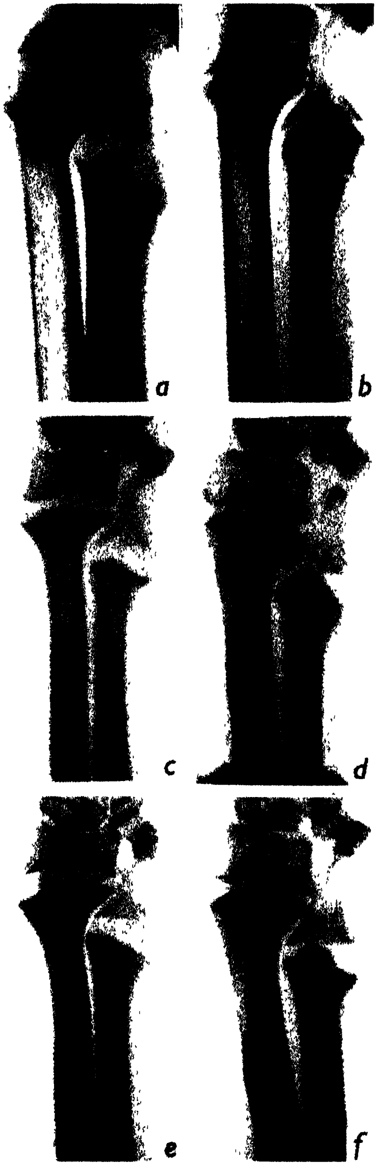

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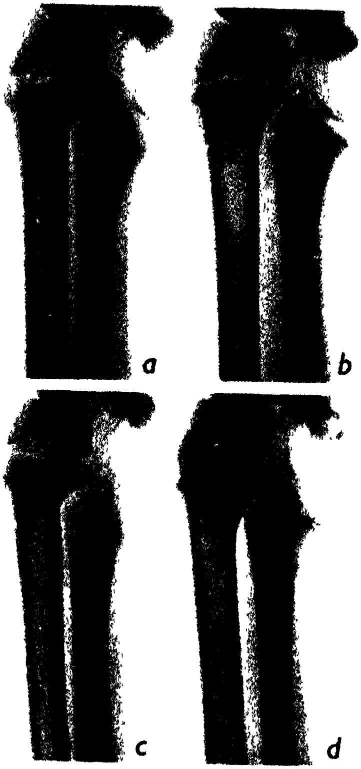
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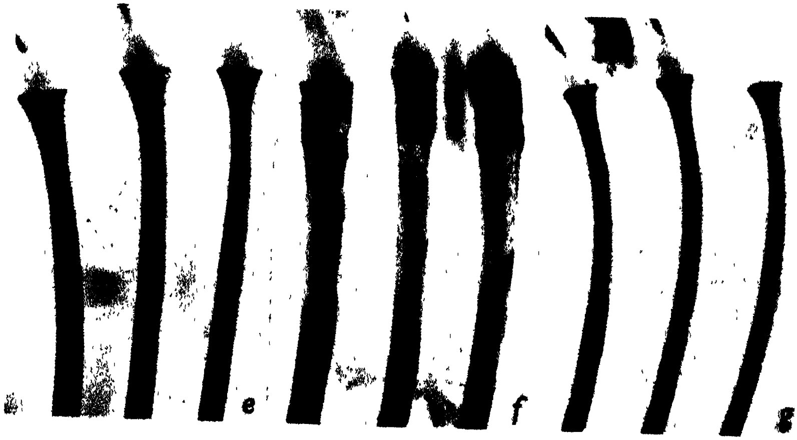
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EXPLANATION OF PLATES

PLATE 1

Exp. 1. (*a*) and (*b*). Radiographs of forepaws at end of experiment, showing relative rickets-producing effect of phytate and phosphate in the presence of high vitamin D reserves. (*a*) Puppy 1. Untreated oatmeal (phytate intact). (*b*) Puppy 2. Oatmeal which had been boiled for 18 hr. with 1% HCl (most of phytate hydrolysed to phosphate). *Note.* Puppy 1 had severe rickets whereas puppy 2 was still normal after 11 weeks.

Exp. 2. (*c*)–(*f*). Radiographs of forepaws showing relative rickets-producing effect of phytate and phosphate in the presence of low vitamin D reserves. (*c*) and (*e*) Puppy 3. Untreated oatmeal (phytate intact). (*c*), 6 weeks on diet. (*e*), 8 weeks on diet. (*d*) and (*f*) Puppy 4. Oatmeal which had been boiled for 18 hr. with 1% HCl (most of phytate hydrolysed to phosphate). (*d*), 6 weeks on diet. (*f*), 8 weeks on diet. *Note.* After 6 weeks on the experimental diets (see (*c*) and (*d*)) puppy 4 (phosphate) had less severe rickets than puppy 3 (phytate), but 2 weeks later the condition of the two puppies was more similar (see (*e*) and (*f*)).

PLATE 2

Exp. 3. (*a*)–(*d*). Radiographs showing the relative rickets-producing effect, in the presence of high and medium vitamin D reserves respectively, of oats before and after part of their phytate had been hydrolysed by germination and autolysis. (*a*) Puppy 5. Oats before treatment (phytate intact); high vitamin reserves. (*b*) Puppy 6. Oats after treatment (phytate hydrolysed to phosphate); high vitamin reserves. (*c*) Puppy 7. Oats before treatment (phytate intact); medium vitamin reserves. (*d*) Puppy 8. Oats after treatment (phytate hydrolysed to phosphate); medium vitamin reserves. *Note.* There is a greater difference in the degree of rickets between (*a*) and (*b*) (high vitamin reserves) than between (*c*) and (*d*) (medium vitamin reserves) after 17 weeks on diet.

Exp. 9. (*e*)–(*g*). Radiographs of costochondral junction. (*e*) Puppy 27. Received phosphate with 20 i.u. vitamin D_2. (*f*) Puppy 28. Received phytate with 20 i.u. vitamin D_2. (*g*) Puppy 32. Received phytate with 1000 i.u. vitamin D_2. *Note.* (1) Substituting phytate P (*f*) for equal amounts of inorganic P (*e*) resulted in thicker and more osteoporotic bones and reduced mineral ash content (see Table 9); (2) increasing vitamin D_2 from 20 (*f*) to 1000 i.u. (*g*) reduced the bulk and improved the quality of the bone although the mineral ash content was not increased (see p. 508 and discussion p. 520).

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

17–18 *December* 1948

Intragastric feeding of rats. By G. H. LATHE

The Department of Biochemistry, University of Oxford

Paired feeding of animals is frequently used in experiments where the nutritional status is of considerable importance. It has the disadvantage of being laborious, and, more fundamentally, it involves an element of underfeeding for one animal of the pair, when the other refuses food. As an alternative intragastric feeding may be used. It has been possible to reduce the labour involved in this, and to remove most of the uncertainties in food preparation and feeding.

Composition of diet

The diet must be fluid, homogeneous, and of small volume, if it is to be given in two feedings per day. In our experience this cannot be achieved by using starch and casein as the only sources of calories. Such a diet tends to produce diarrhoea, though it is possible that with frequent feedings this might be overcome. Diets in which fat or sucrose partly replace starch are satisfactory. The composition of a 25 % (of solid matter) fat diet was Casein (AE, Glaxo) 33 g.; arachis oil 42 ml.; cod-liver oil, 4 ml.; wheat starch (selected for suitable viscosity) 107 g.; salt mixture 3 g.; Agar pulverized 4 g.; vitamin supplement 6 ml.; water 176 ml. Total volume 336 ml. It contains 2·7 calories per ml. The preparation of the diet requires attention to certain details, and the following procedure, used routinely, has been found to be uniformly satisfactory. The casein, starch and ground agar are individually sieved (60 to the inch), and the salt mixture finely pulverized in a mechanical mortar. These can be done in bulk. The casein, starch, salt and agar are weighed, and the powders loosely mixed. The water, at 0° C., is poured into a Waring blender (or alternatively a 'Turmix' kitchen mixer, available from Crittall Kitchen Equipment Co. Ltd., London, W. 1), and the vitamin supplement added. The motor is turned on and the powder is poured in over a period of 10–15 sec. After the mixture is relatively uniform the oil is slowly added. During the mixing the temperature rises, and if it is allowed to continue the starch swells and the diet is too viscous.

The mixing should last only 2 min. The final product is then poured into a beaker. It separates slowly and requires stirring by hand before feeding. It becomes thick if stored in a refrigerator.

Method of feeding

The food is administered from a 10 ml. syringe with a metal plunger. The all-glass type provides too much resistance. A large needle, gauge 19, is inserted into a tube made from fabricated 'teliothene', a plastic of the polythene type (obtainable from the Telegraph Construction and Maintenance Company, Telco Works, Greenwich, S.E. 10). These tubes, of 2·5 mm. outer diameter, provide a large inner diameter, and seldom block. The free end is rounded in the edge of a flame.

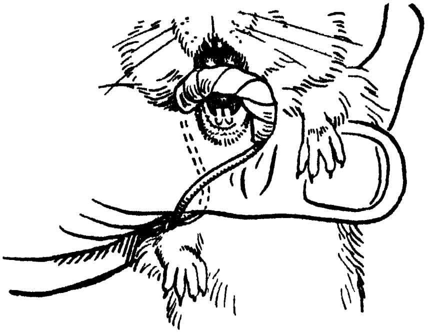

Fig. 1.

The rat, of 200–300 g., is fed with the help of a tape-covered wire ring about the proximal phalanx of the left thumb. The rat is first grasped in the right hand, the right thumb being passed between the forelegs on to the neck and base of the mandible. The ring is slipped over the lower incisors and the animal transferred to the left hand (Fig. 1). It is held with the mouth open, and the neck flexed. The ring should be bent so that it presses back against the mandible and does not exert traction on the lower incisors. The animal is held lightly around the head with the thorax free.

With the right hand the tube is momentarily dipped in water at about 60° C., and the tip is lubricated by dipping it into a small vessel of paraffin. The tube is passed beside the incisors into the back of the throat. Two points of increased resistance are felt. One when it passes into the oesophagus, and the second when the cardia is reached. It may be necessary to pause momentarily when the resistance of the cardia is felt, to avoid damage to the oesophagus. As soon as the tube has entered the stomach (the point may be marked on the tube by a small band) the needle is attached to the syringe which is discharged by pressing the plunger against the chest. With practice one person may feed 30 rats in half an hour. The animals are ordinarily fed twice a day, at nine in the morning and five in the afternoon. The calorie requirements for maintenance of weight may be calculated from the data of Swift & Forbes (1939), and Swift (1944).

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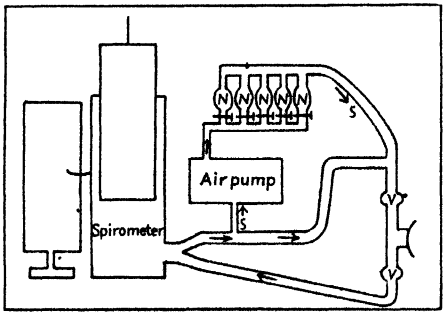
PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
26 *March* 1949

Respiratory recording of asthmatic attacks induced and terminated artificially.

By H. HERXHEIMER. *University College Hospital, London*

A recording spirometer of the Benedict-Roth type is connected to an oxygen cylinder and flowmeter which permits a continuous inflow of oxygen at the same rate as the oxygen in the spirometer is used up. A side circuit (*S*) is attached to the inspiratory tube of the spirometer. It leads into an air pump, and from there into a number of glass nebulizers (*N*) filled with different inhalants. From here the air passes back into the inspiratory tube of the main circuit. The nebulizers are closed by pinchclips and connected to the side circuit by two manifolds which enable them to be used singly and in quick succession. If the pinch clip closing a nebulizer is opened and the pump is switched on, aerosol is emitted into the inspiratory tube and inhaled by the subject. During the expiratory phase aerosol cannot pass the respiratory valves (*V*) and passes back to the pump.



By this method bronchial spasm can be induced if histamine, acetylcholine or an allergenic extract is used as an aerosol, and the attack can be terminated quickly by changing over to an antihistaminic or antispasmodic substance such as pyribenzamine or aleudrine, if a sufficient dosage is used. The disadvantage of the method is that the exact amount inhaled cannot be measured, as the subject exhales a part of the inhaled aerosol into the circuit. The advantage lies in the fact that the substance in question is administered locally and not systemically, and that systemic effects are therefore secondary to the local action. Another advantage is that the inhalation can be broken off at the slightest sign of bronchoconstriction, whilst in parenteral injection the risk of overdosage cannot be avoided. In view of the great sensitivity of asthmatics to histamine this risk is considerable.

The measurement of changes in human limb-volume by means of a mercury-in-rubber strain gauge.

By R. J. Whitney. *Army Operational Research Group, attached to M.R.C. Climatic Research Unit, Department of Human Anatomy, University of Oxford*

The method described measures directly changes in limb girth. For practical purposes, and with parts of the limb of roughly circular cross-section, it can probably be assumed that percentage change in volume is twice percentage change in girth. The apparatus used is much less cumbrous, and is more adaptable than the conventional human plethysmograph.

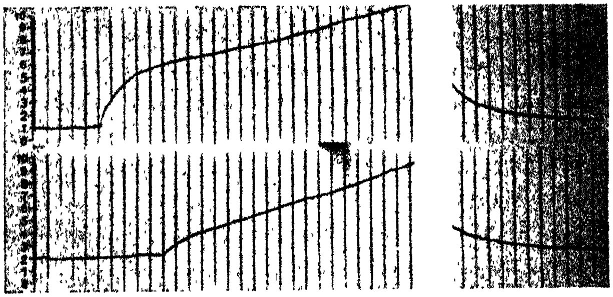


Fig. 1. Examples of records obtained using the gauge for blood-flow determination by the occlusion method. Both records were obtained from the right forearm. Vertical time markings at 6 sec. intervals. Vertical scale is in cm., 1 cm. being equivalent to a change of 1·4 c.c. in 305 c.c. of the forearm. The slow and fast blood-flow rates (c.c./min./100 c.c. tissue) are respectively 3·9 and 15·2.

The pick-up, which is mounted on the part of the limb to be investigated, consists of an easily extensible band, 6 cm. wide, provided with Perspex end-pieces. The band completely encircles the limb and is secured by two screw fittings on the end-pieces, the screws providing a precise adjustment of the tension of the band. The band itself consists of a continuous length of thin-walled rubber tubing, of about 0·5 mm. bore, which is looped between the two end-pieces, the turns of the loops being accommodated in semicircular cavities cut into the end-pieces and the ends of the tubing being securely fixed at the extremities of one of the end-pieces. The extensible band is thereby formed of eight strands of the rubber tubing. The tubing is completely filled with mercury and the ends plugged with tapering brass pins to which the two electric leads are attached. The band therefore forms a mercury-in-rubber resistance strain gauge, and extension or shortening of the band will give a corresponding increase or decrease in its electrical resistance. Precise calibration shows that the response of the gauge is very linear and is constant over long periods.

The changes in resistance of the gauge are recorded continuously by making the gauge one element in a Wheatstone bridge circuit, the bridge being powered by a 2, 4 or 6 V. accumulator, and its output connected directly to a recording galvanometer. Amplification has not been found necessary.

The sensitivity of the method, even employing comparatively insensitive galvanometers, can be much greater than is necessary for certain physiological measurements. An example of the greatest sensitivity which has been found necessary will be quoted. A gauge constructed for measurements of forearm or ankle (25 cm. long, 6 cm. wide, eight strands, resistance 3·5 ohms) is used with a 4 V. bridge circuit and a galvanometer of sensitivity 1·5 mV./cm. of record. With this arrangement 1 cm. of the record is equivalent to a 0·125 % change in the girth of the limb, or to a 0·25 % change in volume. Since the volume of the limb under the gauge is about 300 c.c. the plethysmographic sensitivity is 0·75 c.c./cm. of record. For measuring blood-flow by the occlusion method less sensitivity must be employed if the record is to be made on 120 mm. recording paper. Fig. 1 shows an example of the record obtained from the gauge for blood-flow measurement.

Up to the present the method has been used satisfactorily for determining blood-flow, and for studying the changes of limb volume in relation to water intake. The gauge will also record muscular movements, and it is proposed to investigate its potentialities in this direction.

An electronic stimulator. By V. H. Attree.

Biophysics Research Unit, University College, London

An oscillator circuit operates a fast-acting relay. The relay has change-over contacts, independent of earth, enabling exponential stimuli to be obtained from a conventional battery, condenser and resistance circuit. The oscillator will run at any frequency in the range 0·01 to 1000 cyc./sec. (i.e. 5 decades). Provision is made for external synchronization, either from a pair of contacts or from the supply mains.

The oscillator also drives a delay-circuit which, in turn, triggers a square-wave generator. The time-delay is variable from 100 μsec. to 100 msec. (3 decades) and the pulse width from 50 μsec. to 50 msec. (3 decades also). The output pulse is positive going with respect to earth and has a maximum amplitude of 100 V. The output impedance, which is dependent on output level setting, in no case exceeds 1000 ohms.

Another time-delay and square-pulse generator, identical with that already described, provides a second, independent, square-pulse.

In the demonstration a slow-sweep time-base, developed for general physiological recording, is used to display a typical exponential pulse (from the relay) and the two separate rectangular pulses.

High-speed relays. By D. BAXTER. *Biophysics Research Unit, University College, London*

High-speed relays designed by R. E. H. Carpenter and produced by the Telephone Manufacturing Company have various applications in physiology. They provide simple clean make and break contacts, and are operated by currents of the order of 1 mA. A mechanical make without chatter has been difficult to produce, and for precise work physiologists have usually been forced to employ break contacts only. The accuracy of repetition and rapidity of transit are further advantages.

These relays may be driven by a simple oscillator, and some types will work at frequencies of several hundred cyc./sec. Two or more relays will operate from a single key within less than 0·1 msec. of each other.

An example of their use is seen in the demonstration by B. C. Abbott in which a two-pole relay is used for switching a single beam cathode-ray tube to give double beam facilities.

A simple stimulator designed by V. H. Attree and employing a Carpenter relay in place of a commutator will be shown.

The heat of shortening of muscle. By B. C. ABBOTT and A. V. HILL *Biophysics Research Unit, University College, London*

When a stimulated muscle shortens it gives out extra heat during the shortening proportional to the distance shortened. The effect can be exhibited as follows. The heat production and the shortening are displayed simultaneously on a cathode-ray tube. Contraction is initially isometric, but after an interval the opening of a key releases a magnet and the muscle is allowed to shorten at nearly its maximum speed. A small viscous load is provided (a light chain hanging in silicone fluid DC 200) to obviate chatter due to the sudden release. During the shortening the rate of heat production is largely increased.

The resting tension of muscle.

By A. V. Hill and J. L. Parkinson. *Biophysics Research Unit, University College, London*

The resting tension of an isolated sartorius of frog or toad, in Ringer's solution at its original natural length in the body, is about 30–40 g. wt./cm.^{2} of cross-section, i.e. about 2% of the force exerted in a maximal isometric tetanus. The 'natural length' is measured with the legs gently pulled out in line. The tension diminishes as the length is reduced; to about one-third for a 10% reduction of length. It is measurable down to 60–70% of the natural length, which is less than the minimum length in the body.

The tension is measured, to about the nearest 10 mg., by a single 60μ nickel-chrome wire which forms one arm of a resistance bridge connected to a sensitive galvanometer. The muscle is joined to the wire by a vertical chain, and its length is adjusted by moving the tension-recorder up or down on a Palmer screw-stand (D12).

The resting tension at any length can be temporarily increased by previous stimulation at a rather shorter length, and the range of length within which a tension can be detected is increased in the same way. The method would allow the effect of chemical agents on the resting tension of a thin isolated muscle to be investigated.

The absence of lengthening during relaxation in a completely unloaded muscle.

By A. V. Hill. *Biophysics Research Unit. University College, London*

A toad's sartorius fixed at its lower end is connected by a light chain to a sensitive electrical tension-recorder vertically above it. It is stimulated directly, and its isometric contraction is displayed without friction or sensible lag on a cathode-ray tube. The sweep starts at the same instant as the shock, and the initial upstroke gives the end of the latent period. The length of the muscle is adjusted by screwing the tension-recorder up or down.

If the length is reduced so much that the muscle becomes slack the apparent latent period is greatly increased; the latent period is used as a sensitive indicator of slack. The muscle is stimulated by a few shocks at a short length, then the latent period is measured at various lengths in succession from 50 to 100% of its natural length in the body. From about 65% upwards the latent period is practically constant. This shows not only that the muscle as a whole does not lengthen but that its contractile elements do not relax into folds, after contraction. At lengths below about 60%, lengthening does occur in relaxation, as is shown by the large increase in latent period; but this is a secondary effect due probably to an elastic restoring force set up by the excessive bulging of the muscle. Over a range of lengths considerably greater than is possible in the living body a muscle does not lengthen after contracting unless it is pulled out.

It is necessary for this demonstration that the resting muscle should be completely free from external load; a very small force is sufficient to draw it out after contracting at a very short length.

The electric response at a sensory nerve ending. By Bernhard Katz.

Biophysics Research Unit, University College, London

Action potentials are led off from a sensory axon at a point close to its terminals in a muscle spindle of the frog (Fig. 1). The responses so recorded differ in three respects from the ordinary nerve spike. (i) There is a 'negative after-potential' localized at the nerve endings which follows the sensory orthodromic, as well as the antidromic, spike. (ii) If the tension of the muscle is low, random spikes occur at irregular intervals. These impulses are initiated by brief 'pre-potentials'. The pre-potentials recur in two or three discrete steps and often remain abortive. (iii) Stretching of the muscle (Fig. 2) is accompanied by a depolarization of the nerve endings. In the normal spindle the depolarization gives rise to

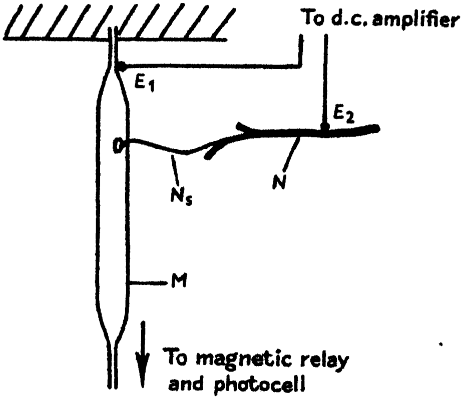


Fig. 1.

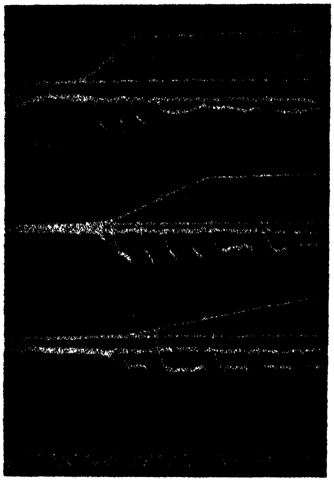


Fig. 2.

Fig. 1. Arrangement for recording potential changes at the muscle spindle of the frog. M, extensor longus dig. IV. N_s, sensory axon. N, peroneal nerve. E_1, E_2, recording electrodes at fixed end of muscle and on the peroneal nerve, respectively. The muscle is stretched by a magnetic relay, and the movement is recorded photoelectrically by the second beam of the oscilloscope.

Fig. 2. Electric responses (lower trace of each pair) during three mechanical stretches at different rates (upper traces). The response consists of spikes and of a slow local depolarization which depends upon rate and amplitude of stretching. Time signal: 500 cyc./sec.

repetitive spikes. After treatment with a local anaesthetic, the spike discharge disappears, but the local depolarization in response to stretching remains. The intensity of this depolarization is a function of the velocity as well as of the amplitude of stretching.

A galvanometer of high volt-sensitivity. By A. C. Downing.

Biophysics Research Unit, University College, London

For many purposes a galvanometer of high volt-sensitivity and reasonably short deflexion time is required. The usual type of sensitive galvanometer is designed for approximately critical damping, so that its deflexion time is about equal to its period. The instrument shown disregards the period and accepts considerable overdamping, aiming only at a time constant of the (very nearly) exponential deflexion which is short enough for the purpose in hand. This allows the construction to be greatly simplified.

The time constant T of the deflexion is related to the microvolt sensitivity S (mm./μV. of a reflected image at 1 m. distance) by the equation $T = \frac{1}{3}SAH \times 10^{-5}$, where H is the field (gauss) and A is the total area of the coil (area of one turn in cm.^{2} × number of turns). In the instrument shown, designed for a low external resistance, the internal resistance is about 20Ω, A is about 1·5 cm.^{2}, H is about 8000 gauss and the maximum value of S (with zero external resistance) is about 20 mm./μV. T therefore is 1·2 sec. and the deflexion is effectively complete in 5 sec. With 15Ω external resistance S and T are halved.

The coil is suspended above and below, no clamp is required for transit and accurate levelling is unnecessary.

A galvanometer designed for a high external resistance would have a larger number of turns, a greater value of A and a higher internal resistance.

The depolarizing action of acetylcholine on muscle. By Paul Fatt.

Biophysics Research Unit, University College, London

As previously shown by histological and electrical methods (Pézard & May, 1937; Eccles, Katz & Kuffler, 1941), the motor nerve endings in a frog sartorius muscle are distributed in a characteristic pattern, the proximal eighth of the muscle being devoid of end-plates, with the latter occurring in greatest density on either side of the nerve entry.

This distribution is demonstrated by the steady depolarization produced after bathing an isolated sartorius preparation in eserine-Ringer's solution containing 1–2 × 10^{-6} Ach. After soaking for a minute or two the solution is drained away and potentials are recorded between two agar wick electrodes leading off to calomel half-cells. With one electrode fixed at the pelvic end, the other is moved along the length of the muscle.

The focus of maximum end-plate density having been located (as indicated by maximum depolarization), the moving electrode is now placed on that point. The muscle is washed in an Ach-free medium of varied ionic composition and the local effect of Ach is tested again by applying it to the end-plate focus. The resulting potential change is recorded via a d.c. amplifier on a smoked drum together with the mechanical response.

The depolarization due to Ach is greatly reduced by soaking the muscle in a solution in which NaCl has been totally replaced by an osmotically equivalent amount of glucose, KCl (0·015%) and $CaCl_2$ (0·020%) remaining as in normal Ringer's solution. The recorded potential changes are very sensitive to the addition of small amounts of NaCl. NaCl in a concentration of 5·8 mM. (5% that in Ringer's solution) is sufficient to increase the depolarization to well above that found in its absence. If KCl and $CaCl_2$ are maintained as in normal Ringer's solution the results are reversible over several hours.

The mechanical response to Ach is lacking in the absence of NaCl, as was shown for the perfused gastrocnemius by Cicardo (1938), and does not begin to reappear until about 10% of the NaCl of Ringer's solution has been restored. This is in contrast to the contraction evoked by KCl (20–50 mM.) which is seen in both the presence and the absence of NaCl. The removal of NaCl in this case results only in the abolition of sporadic twitching due to the irregular firing of impulses in groups of muscle fibres.

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Liberation of noradrenaline from adrenal medulla by splanchnic stimulation.

By EDITH BÜLBRING and J. H. BURN. *Department of Pharmacology, University of Oxford*

It is well known that denervation of the cat's nictitating membrane makes it more sensitive to the action of adrenaline. The height of contraction of the denervated membrane is commonly 1·5–2·0 times as great as that of the normal membrane. When noradrenaline is injected we have found that this ratio is much greater, from 4·0 to 20·0. In spinal cats, after evisceration and exclusion of the kidneys, stimulation of the splanchnic nerve produces contractions of the nictitating membranes. The ratio of these contractions indicates that the discharge from the adrenal medulla is usually a mixture of noradrenaline and adrenaline.

The role of carbamino compounds of haemoglobin in the respiratory transport of CO_2 by the blood. By F. J. W. ROUGHTON.

Department of Colloid Science, University of Cambridge

Ferguson & Roughton (1934, 1936) and Stadie (1937) estimate that these compounds account for 18–30% of the CO_2 eliminated during respiration. On Wyman's (1948) recent interpretation:

(i) $[HbCO_2]/[Hb] = \theta$ (constant) $pCO_2/[H^+]^2$,

(ii) θ_{RedHb} is almost the same as θ_{O_2Hb}, the extra carbamate of RedHb solutions being entirely due to more alkaline pH,

(iii) the role of $HbCO_2$ in CO_2 transport is only about 10%.

Wyman's suggestions are open to several criticisms, and in any case only indicate *minimum* values for the role of $HbCO_2$. Furthermore, he apparently over-estimates the pH difference between O_2Hb and RedHb; using more direct methods I calculate (1949) that $\theta_{RedHb}/\theta_{O_2Hb} = 2{\cdot}0\text{–}2{\cdot}2$, and on combining this result with the average of Ferguson's data (vide 1936, Fig. 3), I confirm approximately the estimate of the role of $HbCO_2$ given by Ferguson.

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Variation in the acetylcholine content of the brain with the physiological state. By JAMES CROSSLAND and DEREK RICHTER.

Neuropsychiatric Research Centre, Cardiff

Acetylcholine was estimated in the rat brain after freezing the brain *in situ* with liquid air (Richter & Dawson, 1948). Acetylcholine was extracted with acidified eserinized Ringer solution at 0° C. and estimated with the frog rectus and leech muscle preparations.

The acetylcholine content in series of rats under nembutal anaesthesia (mean 1·75 μg./g.) and in rats taken in the sleeping state (1·41) was significantly higher than in normal litter-mate controls (1·15). Rats taken during emotional excitement produced by withdrawal of support (0·97) and during electrically induced convulsions (0·54) gave a lower brain acetylcholine content. Tobias, Lipton & Lepinat (1946) have previously reported an increase in the acetylcholine content of the brain in anaesthesia.

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The relation between serum cholesterol and body build in healthy young men.

By J. M. TANNER. *Anthropological Laboratory, Department of Human Anatomy, University of Oxford, and Sherrington School of Physiology, St Thomas's Hospital Medical School, S.E.* 1

The relationship between serum cholesterol level and physique has been investigated on forty-six healthy male medical students. Total cholesterol was estimated by the method of Schoenheimer & Sperry (1934) and physique assessed by Sheldon's somatotypes (1940). Each subject's blood was taken on two occasions a week apart, the correlation between the two cholesterol levels being 0·945.

Cholesterol is significantly related to endomorphy, with $r=0{\cdot}38$. The regression is linear, accounts for 15% of the inter-individual difference in cholesterol level, and is expressed by

$$\text{Cholesterol (mg./100 ml.)} = 11 \text{ endomorphy} + 154,$$

with a standard error of estimate of 27. This equation can be directly used to refine somewhat the present clinical standards. There is no direct relation with mesomorphy or ectomorphy. A relation of similar magnitude exists between cholesterol and the thickness of various subcutaneous fat folds, but the regressions are somewhat curvilinear.

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The movements of radioactive sodium during nervous activity.

By R. D. KEYNES. *Physiological Laboratory, University of Cambridge*

The rates of passage of radioactive sodium and potassium through nerve membranes have been studied in single *Sepia* axons (200μ diameter), using a method similar to that described previously (Keynes, 1948). The rate of entry of Na^{24} was increased about 15 times by stimulation at 100 impulses/sec., the total inward movement during activity being 10×10^{-12} mol./cm.^{2}/impulse. The net entry of sodium must have been less than this, since stimulation also caused a marked increase in the outward sodium flux, but its exact size could not be determined. This observation does, however, indicate that the sodium permeability of the membrane increases considerably during activity, and supports the ideas of Hodgkin & Katz (1949) concerning the part played by sodium in nervous conduction. In these fibres the outward movement of potassium was 5×10^{-12} mol./cm.^{2}./impulse, while only one-tenth as much moved inwards.

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Cardiac output and the peripheral circulation in malnourished subjects (Wuppertal, B.A.O.R., 1946). By SHEILA HOWARTH.

Department of Physiology, University College, London

Twenty subjects were studied by cardiac catheterization and venous occlusion plethysmography. All had bradycardia and either exhibited or reported recent famine oedema; half the group had subnormal blood pressures. The basic food ration, doubtless supplemented, was 1050 cal./day (35 g. protein, 15 g. fat and 176 g. carbohydrate).

Resting cardiac output was variable, most commonly slightly subnormal. Oxygen consumption was usually reduced, while arterio-venous oxygen differences were frequently diminished. Arterial oxygen saturation was normal. Right auricular pressure was raised in half the cases. Raising or lowering auricular pressure by infusion or venesection produced changes in cardiac output which were small considering the magnitude of pressure change. Increasing heart rate by atropine injection produced a normal increase in cardiac output.

Resting blood flow through the hand was conspicuously reduced. Forearm blood flow was also reduced below normal values. Responses in forearm blood flow to arterial occlusion, feet heating and intravenous infusions appeared qualitatively normal.

Phosphate excretion in man. By M. GRACE EGGLETON and Y. A. HABIB.

Department of Physiology, University College, London

Of the factors responsible for the varying rate of phosphate excretion in man, little attention has been paid to the possible effect of changes in glomerular filtration rate (G.F.R.). In a series of experiments large fluctuations were induced, usually by ingestion of ethyl alcohol, in G.F.R. (determined by creatinine output), pH, and rate of urine flow. The phosphate output was most frequently correlated with G.F.R., to a less extent with pH and never with rate of flow. Such correlations were observed in 82% of the 102 comparisons made (in sixteen experiments on nine subjects): in 33%, an increased phosphate output accompanied a rise in G.F.R. and fall in pH or vice versa, in 34% the effect of changing G.F.R. predominated, and in 15% that of pH. The former was statistically significant, the latter was not.

The effect of exercise on the latent period of water diuresis in man.

J. A. BARCLAY, R. J. CLARKE and A. E. SLATER. *Department of Physiology, University of Birmingham*

It has been shown that pituitrin given with water lengthens the latent period of a water diuresis (Barclay & Kenney, 1949). Exercise immediately following the ingestion of water gave a latent period of 30 min. (23 subjects). The latent period in a control series was 26 min. (38 subjects).

REFERENCE

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The depression of the demarcation potential of cat's tibialis by bistrimethylammonium decane diiodide (C 10).

By G. L. BROWN, W. D. M. PATON and M. VIANNA DIAS. *National Institute for Medical Research, Hampstead, London*

Demarcation potentials (Brown & Goffart, 1948) and twitch tension of tibialis in cats anaesthetized with chloralose were recorded. After arterial injection of 2μg. C10 (Paton & Zaimis, 1948), demarcation potential fell by 30–50%, rapidly for 10–15 sec., then slowly to a minimum at 5–10 min., recovering in 20–30 min. *pari passu* with the neuromuscular paralysis. Similar falls followed 30–40μg./kg. C10 intravenously. No dose, however big, caused a depression exceeding 60%. Small doses, producing only potentiation of muscle twitch, also elicited a small fall in demarcation potential. At least 5 mg. KCl intra-arterially was required to produce a comparable maximum depression of demarcation potential.

Large doses of C5 arterially or intravenously had no action on demarcation potential, and only slightly antagonized depression of demarcation potential by C10, although the twitch tension of the paralysed muscle rapidly returned to normal. D-Tubocurarine chloride caused a small increase in demarcation potential (about 5%); eserine and prostigmine had no significant effect.

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The penetration of some sulphonamides into the intra-ocular fluids.

By H. DAVSON. *Ophthalmological Research Unit (Medical Research Council), Institute of Ophthalmology*

The rates of penetration ($K_A \times 100$; Davson & Quilliam, 1940), into the aqueous humour of the rabbit, of sulphapyridine, sulphanilamide, sulphadiazine and sulphathiozole are 99·5, 96, 48·4 and 30·0 respectively; rates of penetration into the vitreous body ($K_V \times 100$) are 29, 31, 9·7 and 5·3 respectively. The relatively high rates of penetration of sulphapyridine and sulphanilamide are probably accounted for by their low degrees of dissociation at blood pH; it would appear that the lipoid solubility of the undissociated compounds is also a factor in penetration.

REFERENCE

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Studies on the transfer of *l*-ascorbic acid and dehydro-*l*-ascorbic acid into the eye of the rabbit and cat.

By M. LANGHAM. *Ophthalmological Research Unit (Medical Research Council), Institute of Ophthalmology*

In the rabbit, raising the blood concentration of dehydro-*l*-ascorbic acid (DHA) leads to a marked increase in the *l*-ascorbic acid (AA) concentration in the aqueous humour; however the concentration of DHA in the aqueous humour during this time remains unchanged.

A ceiling level of AA in the aqueous humour (*c.* 50 mg. %) results from either a raised plasma AA or DHA concentration; with continued rise both compounds diffuse into the aqueous humour down the concentration gradient.

In the rabbit with one aphakic eye, kinetic studies show that the ceiling level of AA attained in the aphakic eye is always much less than in the normal eye, although the DHA concentration is always higher than in the normal eye.

It is suggested that accumulation of AA in the aqueous humour of the rabbit involves the reduction of DHA by the lens and other tissues of the eye.

Similar kinetic studies on the cat lend support to the above conception.

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A relation between a psychomotor response and the phase of the alpha rhythm.

By J. L. Boreham, G. O. Kibbler and D. Richter. *Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff*

Simultaneous recordings were made of the alpha rhythm and eye opening in response to an auditory stimulus, using a standard E.E.G. apparatus. By plotting the number of responses against the phase of the alpha rhythm a curve was obtained which showed a variation of 2 to 1 in the frequency of responses in different parts of the cycle.

Similar results were obtained with voluntary actions such as tapping a key in the absence of a stimulus.

The distribution of the responses was further studied by means of an electronic timing device. This apparatus registers a point in the alpha cycle by a signal of 0·5 msec. duration, while the eye opening produces a similar signal. The time interval between the signals is measured to an accuracy of ± 2 msec. by recording the charge gained by a condenser. The observations confirmed that the probability of a response was not randomly distributed in time, but showed peaks and troughs recurring at approximately 10/sec. and in constant phase relation with the alpha rhythm.

Direct observation of stream lines in the basilar artery.

By D. A. McDonald and J. M. Potter. *Department of Physiology, St Bartholomew's Hospital Medical College*

In a previous communication (McDonald & Potter, 1949) we presented indirect evidence that the two blood streams, entering from the vertebral arteries, remain distinct for a varying distance in the basilar artery of the rabbit.

We have now confirmed by direct observation in the living animal that this does occur under relatively normal conditions of blood flow.

The proximal half of the basilar and its junction with the vertebral arteries are exposed in the living rabbit, and the flow of injected dye is studied through a dissecting microscope.

Intravenous Nembutal and open ether anaesthesia are used. A right-angled glass tube is inserted into the trachea low in the neck, and the trachea and oesophagus are divided cranial to this and reflected anteriorly. This exposes the retropharyngeal space. The ventral arch of the atlas, odontoid process, and the basal part of the occipital bone are removed. Judicious use of haemostatic gauze and ligation control otherwise troublesome bleeding from the longitudinal venous plexuses adjacent to these structures.

The ventral atlanto-occipital membrane, dura and arachnoid matres are then incised to expose the arteries on the ventral aspect of the medulla oblongata.

An axillary artery is then exposed and a cannula inserted pointing towards the heart. A small quantity of dye (5% Evans Blue) is injected, and this is carried up the corresponding vertebral artery. It is seen to enter the basilar artery, but remains in a stream of its own and fills only the ipsilateral branches of the artery.

If a thread is passed around the origin of the opposite vertebral artery (this can be done through the axilla), gentle traction on it will occlude the artery. If this is done, and the dye again injected as before, the stream of dye now occupies the whole lumen of the basilar, and also passes down the opposite vertebral which has been occluded proximally.

If dye is introduced indirectly into one internal carotid artery it does not normally reach the basilar. Occlusion of both vertebral arteries, however, reverses the direction of the blood flow in the basilar artery, and dyed carotid blood can be seen passing down it in a stream.

By thus altering the normal balance between the four main arteries supplying the brain, and by injecting dye into any of them, the resulting variations in blood flow can be seen in the basilar artery.

The indirect injection of dye into the arteries concerned is emphasized. Obstruction of the lumen, uncontrolled injection pressure and trauma to the vessel itself, and the effect of these on the normal blood flow are thereby reduced to a minimum.

REFERENCE

McDonald, D. A. & Potter, J. M. (1949). *J. Physiol.* **108**, 34*P*.

The recording of nerve action potentials through skin in man.

By G. D. Dawson and J. W. Scott.* *Neurological Research Unit, Medical Research Council, National Hospital, Queen Square, London*

It has been found that useful nerve action potentials may be recorded from surface electrodes on the skin over the larger peripheral nerve trunks. These action potentials have been used to measure conduction velocity in the nerves and in investigations of the cerebral responses they provoke. The methods used and typical records will be demonstrated.

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* Nuffield Research Fellow.

Measurement of the latency of cerebral responses after flash stimuli.

By W. A. COBB, G. D. DAWSON and H. B. MORTON. *National Hospital, Queen Square, London*

The method uses a crystal-controlled oscillator and frequency dividers giving time intervals up to 1 sec. The time-scale generator with the associated oscillograph, recording camera and driving mechanism will be demonstrated. Records of responses to flash stimuli will be shown.

Observations on the Cartesian diver microrespirometer.

By J. C. WATERLOW and A. BORROW. *Human Nutrition Research Unit, National Hospital, Queen Square, London*

The Cartesian diver microrespirometer, as developed by Linderstrøm-Lang & Holter (1943), has hitherto been used mainly for experiments on lower organisms. We have investigated its suitabilty for measurements of mammalian tissue metabolism, in order to be able to study human tissues obtained by biopsy. The diver method is about one thousand times as sensitive as the Warburg respirometer, and equally accurate. It is almost as versatile, since it is possible both to mix reagents in the diver, and to fill with any desired gas mixture. The main disadvantage of the method is the difficulty of preventing leakage of CO_2, when this is present in the gas phase.

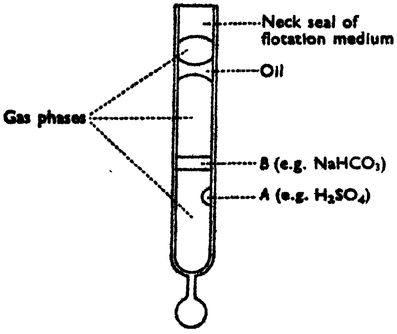

Fig. 1.

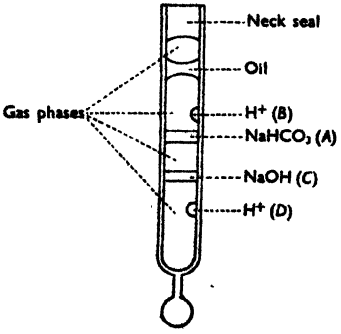

Fig. 2.

Fig. 1. Simple mixing.

'*Standard*' *diver dimensions:* area of section, 1·0–1·2 mm.^{2}; total volume, 1·5–20 μl.; gas volume after filling, 10–15 μl.

Fig. 2. Double mixing.

Reagents can be mixed in the diver by the method of Anfinsen & Claff (1947) (Fig. 1), in which a drop of reagent *A* is placed on the wall of the diver, and a seal of reagent *B* above (or below) it. The two are mixed by applying a positive (or negative) pressure. It is necessary to coat the internal wall of the diver with a water-repellent substance. For this purpose we use the silicone DC200. In measurements of the N_2 evolved on mixing hydrazine with potassium iodate, and of the CO_2 evolved on mixing dilute sulphuric acid with sodium bicarbonate, the average recovery was 99·4%, with a mean deviation of ±1% (amounts of gas 0·2–0·6 cu.mm.).

Double mixings are also possible. Experiments were done under conditions similar to those of R.Q. determinations by the first method of Dickens & Simer (1931) (Fig. 2). CO_2 was evolved from sodium bicarbonate (*A*) by mixing with dilute acid (*B*). It was absorbed by sodium hydroxide (*C*) and released again by mixing with dilute acid (*D*). The average recovery (four experiments) was 98·2%.

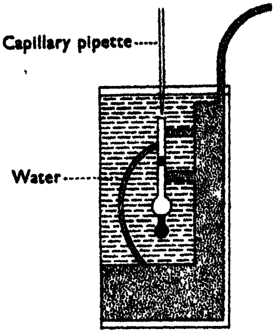

Fig. 3. Diver-holder for gas-filling.

Coating with silicone makes possible a simple method of filling the diver with gas mixtures other than air. The principle of the method is that the operation of filling the diver with gas, and the addition of reagents is carried out under water (Fig. 3).

When the gas mixture contains CO_2, leakage of CO_2 can be reduced by saturating the flotation medium with the gas mixture, and by putting glass stoppers in the diver mouth, as recommended by Linderstrøm-Lang & Holter. That such measures are effective was shown by the results of measurements of plasma choline esterase in the diver and in the Warburg respirometer. The gas phase contained 5% CO_2. The results were:

Warburg respirometer	(mean of 3 measurements)	44·4	cu.mm. CO_2 per ml. plasma per min.
Diver microrespirometer	(mean of 5 measurements)	44·6	

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Changes in the liver of the rat after partial hepatectomy. By R. D. Harkness. *Department of Physiology, University College, London*

Changes in extravascular and blood space in rat liver 10, 24, 48 and 72 hr. after removal of two-thirds of this organ have been investigated. The total quantity of blood in the liver stayed approximately constant till 48 hr., but had risen at 72 hr. This suggests that expansion of the vascular bed lags behind growth of hepatic cells, which starts less than 24 hr. post-operatively.

In addition, wet weight, water content, total fatty acid, glycogen, nitrogen, number and nuclear volume of hepatic cells were investigated. Findings of interest were:

(1) Rise in total fatty acid concentration was already maximal 10 hr. post-operatively.

(2) Concentrations of fat-free glycogen-free solid, and nitrogen, fell to minima 24 hr. post-operatively, and rose subsequently but had not reached the normal value 72 hr. post-operatively.

(3) Nuclear volume of hepatic cells rose more rapidly than cell volume, and stayed up after 24 hr., when cell volume fell.

Pituitary stalk in relation to oestrous rhythm and pseudo-pregnancy in rats. By G. W. Harris. *Cambridge*

The pituitary stalk has been sectioned in fifty-five female rats. The post-operative return of oestrous cycles and pseudo-pregnancy responses may be correlated with regeneration of the hypophysial portal vessels. Rats that remain anoestrous after stalk section show little or no regeneration of the portal vessels. These results support the view that the nervous system controls the activity of the adeno-hypophysis by means of the hypophysial portal vessels (Harris, 1944), and would also suggest that the rhythm of oestrous cycles of the rat originates in the nervous system.

REFERENCE

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The entry of radioactive phosphorus into the phospholipids of the brain. By R. M. C. Dawson (*Beit Memorial Fellow*) and D. Richter. *Neuropsychiatric Research Centre, Cardiff*

The changes in the metabolism of the brain with functional activity *in vivo* were studied by measuring the rate at which P^{32} entered the brain from the blood and was synthesized into the nucleoproteins and phospholipids of the mouse brain. Preliminary experiments showed no significant difference with sex or with age in animals more than 2 months old.

A period of emotional excitement, induced by forced running for 3 hr. in a slowly rotating drum, produced no change in the uptake of P^{32} into the acid-soluble or nucleoprotein fractions of the brain in a series of six animals; but it produced a significant fall of 25 % in the rate of radioactive phospholipid synthesis. This effect was not attributable to concomitant muscular exercise, since it was absent in animals previously conditioned to running in the drum. A similar effect on the phospholipids was produced by direct electrical stimulation of the brain.

Auditory 'adaptation': its experimental investigation in the human subject.

By M. R. Dix, C. S. Hallpike and J. D. Hood. *Otological Research Unit, National Hospital, Queen Square, London*

As is well known, raising of the threshold of hearing is caused by exposure to a continuous tone. The magnitude of this effect is a function of the frequency, duration and intensity of the tone.

The threshold measurements required for the demonstration of this type of fatigue are carried out following the application of the stimulus. It is, therefore, conveniently termed *post*-stimulatory fatigue.

By means of a binaural loudness balance technique it has been possible to demonstrate and investigate a form of aural fatigue which appears *during* the application of a fatiguing tone, and may be termed *per*-stimulatory fatigue. Its characteristics differ in certain important respects from those of *post*-stimulatory fatigue, and make it likely that *per*-stimulatory fatigue is, in some respects, the subjective counterpart of auditory equilibration of which the electro-physiological characteristics in the cat have been described by Derbyshire & Davis (1935).

REFERENCE

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Experimental observations upon the subcortical pathways of optokinetic and vestibular nystagmus.

By M. R. Dix, C. S. Hallpike and M. Spencer Harrison. *Otological Research Unit, National Hospital, Queen Square, London*

Vestibular and retinal excitation both give rise to ocular nystagmus. In each case the nystagmus exhibits a regular alternation of slow and fast movements. This identity is explained by Ohm (1936) and Ter Braak (1936) upon the hypothesis that in both movements a common neural mechanism is involved which they consider to be provided by the vestibular nuclei. According to this theory the destruction of the vestibular nuclei would abolish both types of nystagmus.

It is well known that streptomycin causes vestibular paralysis, in all probability by its selective destruction of the vestibular nuclei, and investigations of vestibular (caloric) and optokinetic nystagmus have accordingly been carried out upon twelve subjects treated with this drug. In all of these, calorie nystagmus was abolished or grossly reduced. Optokinetic nystagmus, however, was brisk and normal in every case. It is necessary, therefore, to conclude that the pathway of optokinetic nystagmus is entirely independent of the vestibular nuclei.

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Action of D-tubocurarine chloride on the cerebral cortex of the cat.

By W. F. Floyd, S. Salama and Samson Wright. *Middlesex Hospital Medical School, London*

D-Tubocurarine chloride was applied directly to the cerebral cortex of the cat, or injected intraventricularly, intracisternally or intravenously. Electrocorticograms were recorded simultaneously from various parts of the two cerebral hemispheres by electrodes in contact with the intact dura. The complex electrical changes observed will be described.

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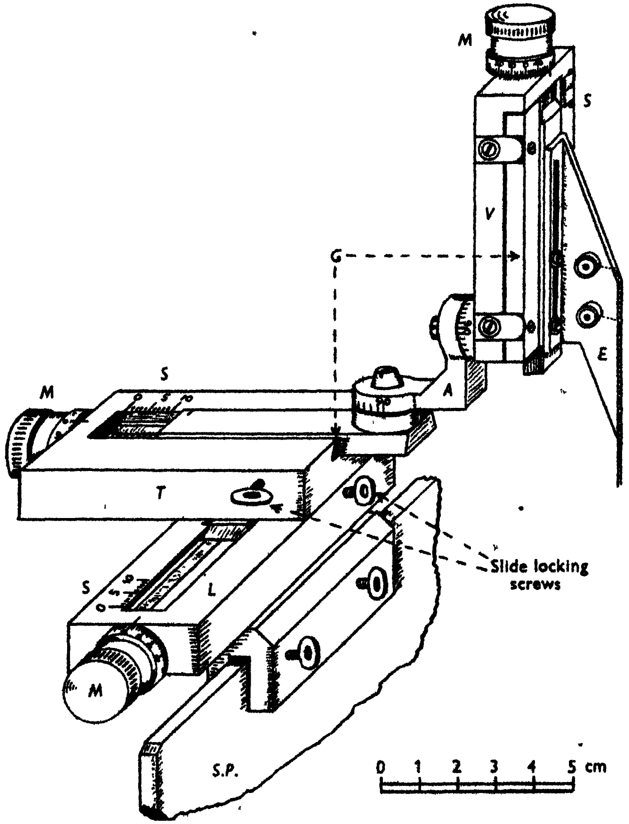
A compact and versatile stereotaxic instrument.

By H. Rosenberg and V. C. Tindley. *Department of Physiology, Royal Veterinary College, London*

Adaptability, rigidity and ease of operation have been the aims in the design of the present stereotaxic instrument which in some respects differs from the classical Horsley-Clarke model.



The head stand and holder and the stereotaxic instrument proper are made almost completely in brass to minimize magnetic fields. The micrometer knobs

(*M*) are graduated in 0·02 mm. divisions. The pitch of the steel micrometer screws is 1 mm., the slideways (*S*) are indexed in mm., and the maximum distance of travel is 30 mm. The transverse (*T*) and the longitudinal (*L*) slides have spring-loaded gibs (*G*) and locking screws. The vertical slide (*V*) has an adjustable spring-loaded gib. This slide can be rotated through 180° in either the sagittal or transverse planes. The angle protractors (*A*) are graduated in 10° divisions.

Rotation about the transverse axis enables the electrodes to be set vertically to any transverse plane or to reach parts difficult of perpendicular access (Harrison). Rotation by 90° about the vertical axis will allow lateral angular insertion, with, for example, 45° inclination. The initial longitudinal setting is measured along the side plate (*SP*).

The Perspex electrode attachment (*E*) shown is for sagittal insertion. Various shapes have been made for different head-size stands and different sets of electrodes. For lateral horizontal introduction a rectangular attachment with horizontal electrode needles is required. The attachments are slotted for initial adjustment.

The stereotaxic unit can be fitted on different head stands constructed for the various types of animals investigated. Two units fixed on either side of the head stand permit of the examination of two loci in the brain simultaneously or in quick succession.

As the shape and size of the skulls of animals, with few exceptions, vary widely, the construction of head holders which enable the electrodes to be present, is generally not feasible, and the position of the tips of the electrodes has been defined with reference to a suitable zero point, such as the obex of the 4th ventricle in the exploration of the medulla oblongata.

The instrument has been successfully used, in collaboration with E. C. Amoroso and F. R. Bell, in the localization of the inspiratory and expiratory regions in the medulla of the cat and goat, and of such regions and also vasomotor sites, both pressor and depressor, in the medulla of the dog and sheep.

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Variations in the zona reticularis of the adrenal cortex of the cat correlated with the sexual cycle.

By M. C. Lobban*. *The Physiological Laboratory, University of Cambridge*

Azan-stained sections of adrenals from adult cats of both sexes show great variation in the proportion of the cortex occupied by the zona reticularis.

Measurements of the widths of the glomerulosa, fasciculata and reticularis in a series of cats show:

(1) The zona glomerulosa and fasciculata are of approximately constant width in all cats.

(2) The fasciculata/reticularis (F/R) ratio (i.e. width of fasciculata/width of reticularis, expressed as percentages of the cortex width excluding the glomerulosa) is variable. If, however, the cats be divided into groups according to the state of activity of their gonads, there is a characteristic value of the ratio for each group.

(3) It is chiefly the variation in width of the reticularis which is responsible for the differences in the F/R ratio.

The zona reticularis in the cat gives similar acidophilic staining reactions to those of the '*X*-zone' (Miller, 1927; Deanesly, 1928) in the adrenal of the mouse.

* With a research grant from the Agricultural Research Council.

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Miller, E. H. (1927). *Amer. J. Anat.* **40**, 251.

The effect of maximal respiratory movements upon intra-abdominal pressure. By J. N. Mills. *Department of Physiology, University of Cambridge*

Rushmer (1946) has shown that intra-abdominal pressure can be recorded in man by means of a balloon in the rectum. It has been found that a gastric balloon, connected to a rubber membrane manometer, presents certain advantages.

During quiet breathing, a trifling rise and fall of pressure occur with inspiration and expiration. Maximal expiratory efforts cause the pressure to rise to between 100 and 200 mm. Hg as the last few hundred c.c. of air are expelled. Maximal inspiratory effort produces a similar rise, but only after air has ceased to enter the lungs.

REFERENCE

Rushmer, R. F. (1946). *Amer. J. Physiol.* **147**, 242.

An instrument for the study of vascular factors in the physiology of the human mammary gland. By V. R. Pickles. *Physiology Department, Medical School, King's College, Newcastle upon Tyne*

A full description of this instrument would be lengthy, but the main features have been shown in the diagram (Fig. 1). In essence it consists of a special breast plethysmograph and a device for periodically lowering the pressure of air therein by a few mm.Hg below atmospheric. During such periods the breast swells. Prominent among the factors causing this swelling is engorgement of the mammary vessels with blood and lymph. When the instrument is correctly adjusted it records a sine curve (with added respiratory and cardiac components) whose amplitude can be shown to be almost exactly proportional to the

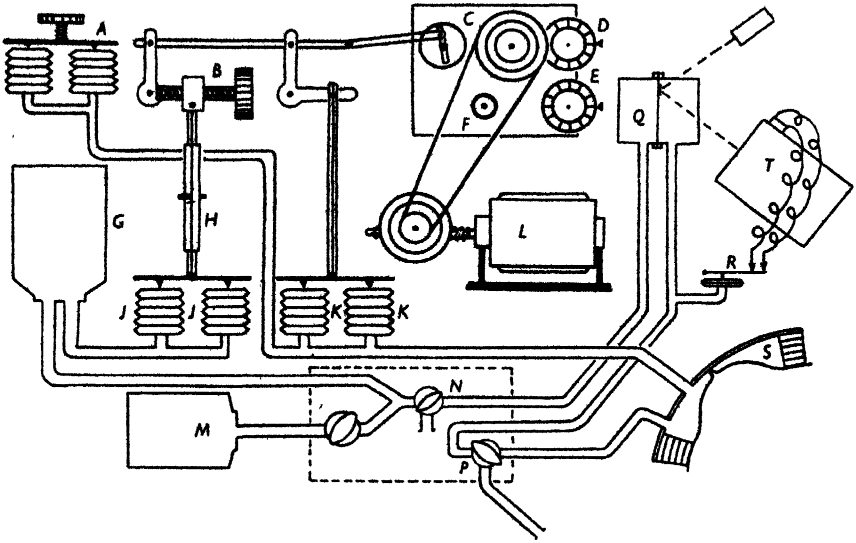

Fig. 1. *A*, air-pressure adjustment to plethysmograph; *B*, stroke adjustment for bellows *JJ*; *C*, variable speed and stroke-crank mechanism; *D*, dial registering phase of cycle of events; *E*, dial registering stroke of crank mechanism; *F*, control for varying stroke of crank mechanism; *G*, rigid bottle; *H*, pressure adjustment for bellows *JJ*; *JJ*, 'Compensator' bellows; *KK*, 'plethysmograph' bellows; *L*, $\frac{1}{4}$ h.p. synchronous motor; *M*, rigid bottle used in calibration, etc.; *N*, *P*, three-way taps for opening tubes to air; *Q*, differential optical manometer; *R*, signal recording plethysmograph air pressure; *S*, special breast plethysmograph containing air; *T*, recording camera.

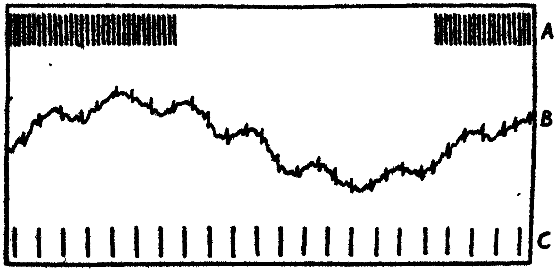

Fig. 2. Copy of section of a typical record. *A*, signal marking plethysmograph air pressure; *B*, sine curve with added components; *C*, time in seconds.

rate of this increase in breast volume at any given subatmospheric pressure (Fig. 2).

Evidence will be given later that this quantity is predominantly determined by the rate of blood flow through the breast and the underlying muscles, and that other physiological factors are generally small in comparison. The instrument is thus a convenient means of investigating both short- and long-term fluctuations in mammary blood flow.

The action of anticholinesterases on striated muscle. By F. Hobbiger.

Pharmacology Department, Middlesex Hospital Medical School, London

The cholinesterase in the frog rectus muscle was completely inactivated by tetraethylpyrophosphate (5×10^{-6} for 30 min.), so that the direct effect of anticholinesterases on the muscle could be assessed. In high concentrations neostigmine and eserine produced some contraction of the muscle which was less than additive with the contraction produced by acetylcholine, nicotine or carbachol, but additive with KCl. On the unsensitized muscle treated with D-tubocurarine (5×10^{-7}), anticholinesterases antagonized the effect of D-tubocurarine only when acetylcholine was used as excitant, and not when nicotine, carbachol or decamethonium iodide were so used. In muscle treated with tetraethylpyrophosphate and D-tubocurarine an anticurare action was no longer found with neostigmine or eserine, even with acetylcholine excitation; in higher concentration some additive effect with curarine sometimes occurred. The anticurare action of neostigmine and other anticholinesterases in this preparation is therefore attributable purely to inhibition of cholinesterase.

Deuteranopia and the sensitivity of the 'red receptor'.

By E. N. Willmer. *Physiological Laboratory, University of Cambridge*

While the central fovea of the normal subject appears to have two receptor pathways, that of the red-green-blind subject has only one. In protanopes (red-blind) this pathway is most sensitive at $\lambda = 540$ mμ. In deuteranopes (so-called green-blind) the maximum sensitivity may range between 560 and 580 mμ. Those subjects maximally sensitive at 580 mμ. have their sensitivities to both red and violet test fields raised to the same extent by either red or violet adapting stimuli of equal brightness (1000 times the threshold strength for the particular subject). This suggests the presence of only one type of receptor and a single pathway (the 'red' mechanism). Those deuteranopes whose maximum sensitivity occurs at 560 mμ. show a pattern of adaptation very like that of the normal subject. They probably have the two receptors of the normal subject, but the pathways fuse before reaching the cortex.

Pituitary gland and blood lymphocytes. By H. F. Colfer, J. de Groot and G. W. Harris. *Physiological Laboratory, University of Cambridge*

Emotional stress in rabbits is followed by a marked drop in the number of lymphocytes in the blood. The effect is maximum at about the third hour after the stress stimulus. This response is abolished by hypophysectomy, although injection of anterior-pituitary extract or purified adrenocorticotrophic hormone still produces a lymphopenia in the hypophysectomized rabbit. This effect of emotional stress on the blood lymphocytes may be used as a rapid indicator of anterior-pituitary activity.

Carotid nerve reflexes and effects of adrenaline in the horse and mule. By E. C. Amoroso, F. R. Bell, A. S. King and H. Rosenberg. *Departments of Physiology and Anatomy, Royal Veterinary College, London*

In Equidae the nerve from the carotid bifurcation joins the glossopharyngeal and, sometimes, also the sympathetic of this region. Branches from the carotid body were not separable from the main trunk.

Under chloralhydrate (initial i.v. dose 0·15–0·3 g./kg.), in all (8) animals, clamping the left common carotid causes a distinct rise and unclamping an equivalent fall of arterial pressure (right common carotid—original pressure 120–130 mm. Hg). Respiratory excitation is small or absent.

Faradic stimulation of the glossopharyngeal component lowers the pressure, slows the heart, and greatly increases respiratory rate and depth. Atropine (60–80μg./kg.), by abolishing cardiac inhibition, diminishes the fall in pressure while the respiratory response is unaffected.

Adrenaline (5–10μg./kg.), while raising the pressure by 50–60 mm. Hg, in contrast to the dog and cat, increases the respiratory depth and/or rate.

The bottom of the oxyhaemoglobin dissociation curve of diluted sheep blood. By W. Paul and F. J. W. Roughton. *Department of Colloid Science, University of Cambridge*

By improvements in gasometric technique we have studied the O_2Hb dissociation curve of sheep blood at very low saturations (0–5% O_2Hb), with a much greater precision (viz. $\pm 0{\cdot}1$% O_2Hb) than heretofore. The initial part of the curve is straight for a short distance, and from the slope of the line, the value of K_1, the equilibrium constant of the first of the four reactions of O_2 with Hb_4, is calculable. The influence of pH, temperature, dilution, etc., on K_1 have been studied, and compared with their effects on p_{50}, the O_2 pressure required for 50% saturation. Contrary to previous indications, these factors have different effects on K_1 from their effects on p_{50}, and it can no longer be held that pH and temperature only affect the scale and not the shape of the dissociation curve. Many current ideas about the nature of the oxyhaemoglobin equilibria therefore require reconsideration.

Antidiuretic activity in the urine of dogs following the administration of sodium chloride. By W. J. O'Connor. *Department of Pharmacology, University of Cambridge*

The response curve of the blue receptors. By H. Hartridge. *Vision Research Unit, Institute of Ophthalmology, Judd Street, London*

It is very probable that the luminosity, at any part of the spectrum, is the summation of the separate luminosity contributions of all the receptors which respond to that part, and it follows that the inactivity of a particular type of receptor will produce a notch in the normal luminosity curve, and that the difference between the ordinate of the notched and the normal curves will indicate the ordinates of the response curve of the inactive receptors.

On this basis the writer calculated the shape of the response curve of the yellow receptors from the notch found by Wright (1942) for test objects of small size. He has now performed a similar calculation for the blue receptors from the notch recently found by Thomson (1949).

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A sensitive biological test for adrenaline. By R. C. King. *St Bartholomew's Hospital Medical College, London*

The isolated uterus of the non-pregnant rat exhibits poor rhythmic activity when suspended in oxygenated Tyrode's solution (0·02% KCl) at 37° C. The rhythm and the amplitude of the contractions are irregular, but both become more regular if the concentration of KCl is increased to 0·15%, the concentration of NaCl being diminished by an equivalent amount.

The addition of 12% KCl, drop by drop, to the fluid bathing the uterus diminishes the amplitude of the contractions by inhibiting full relaxation. When the bath contains about 0·20% KCl, the movements of the uterus are abolished, or nearly abolished, and the muscle remains contracted. This preparation responds regularly, by relaxation, to a bath concentration of 2 in 10^{11} adrenaline, and sometimes a bath concentration of 1 in 10^{11} gives an easily measurable response.

Sensitivity of palmar sweat glands to acetylcholine in essential hyperidrosis. By T. M. Chalmers and C. A. Keele. *Department of Pharmacology, Middlesex Hospital Medical School, London*

In eight normal subjects and in six patients with hyperidrosis of the hands the median nerve at the wrist was blocked by procaine, in order to eliminate nervous control of the palmar sweat glands. The response of these glands to intradermal injection of 0·01 ml. of a solution of acetylcholine chloride was then observed by measuring the change in skin electrical resistance, and by the iodine-starch paper test. The smallest effective concentration of acetylcholine varied from 10^{-3} to 10^{-6} in normal, and from 10^{-4} to 10^{-8} in hyperidrotic subjects. It is concluded that this form of hyperidrosis is not due to an increased sensitivity of the sweat glands to acetylcholine.

The action of decamethonium iodide (C_{10}) on foetal neuromuscular transmission and its transfer across the placenta. By I. Maureen Young. *Sherrington School of Physiology, St Thomas's Hospital, London*

The possibility that C_{10} may be used, as an adjunct to the anaesthetic, to produce relaxation during Caesarian section has made it desirable to investigate (*a*) its action on foetal neuromuscular transmission and (*b*) its placental transfer.

The results were as follows:

(1) Neuromuscular transmission is paralysed in phrenic nerve diaphragm preparations from near term rabbit and guinea-pig foetuses and 18–22-week human foetuses; paralysis is not reversed by prostigmine, K^+ or C_5 (pentamethonium iodide).

(2) C_{10} given intravenously to the mother does not cross the placental barrier in near-term rabbit or guinea-pigs. Foetal activity was recorded by the mass reflex response to a pinch applied to one hind leg.

(3) C_{10} injected into the umbilical artery in doses equivalent weight for weight to 50–100 times a therapeutic dose in man did not pass into the maternal blood stream in near term rabbits or guinea-pigs.

Absorption of acetate from the rumen of sheep. By A. T. Phillipson. *The Rowett Research Institute, Aberdeenshire*

The concentration of acetate in the rumen is normally between 0·03 and 0·13 M and the pH between 6 and 7. It is important to know whether the anion is absorbed (see Barcroft, McAnally & Phillipson (1944) and Grey (1948)).

The rumen and reticulum and omasum, or the rumen and reticulum, were separated from the remainder of the alimentary canal by ligature under nembutal anaesthesia, and solutions of sodium acetate were placed in the empty rumen. The higher concentration of acetate in the venous blood from the rumen showed that absorption occurred, and the arterio-venous difference increased as the concentration in the rumen increased. Absorption from normal rumen liquor made alkaline with bicarbonate was similarly demonstrated.

With three sheep fitted with a rumen cannula 3 weeks or more before the experiment, the loss of acetate from the rumen was 2·53, 2·06 and 3·35 g./hr. (as acetic acid), but in two animals fitted with a cannula immediately before the experiment the loss was only 1·63 and 0·57 g./hr.

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Acetylcholine release and synthesis in the wall of the small intestine.

By W. FELDBERG* and R. C. Y. LIN† (*Chengtu*). *Physiological Laboratory, University of Cambridge*

The acetylcholine of intestinal wall is commonly thought to be derived from the myenteric plexus and associated with activity of muscularis externa. That cocaine does not affect acetylcholine release from intestine may suggest that nervous structures are not the site of acetylcholine metabolism which may be comparable to that in human placenta. Synthesis of acetylcholine occurs also in mucosa and may be associated with activity of muscularis mucosa and secretion of succus entericus. Amounts synthesized (μg./hr./g.) for acetone-dried intestine were: 70–480 guinea-pig, 33–133 rabbit, 35–120 dog; mean values from three dog's small intestine were for mucosa proper, muscularis mucosa, circular and longitudinal muscle 104, 53, 41 and 77 respectively, but it has to be realized that the circular muscle is two to three times thicker than the longitudinal, and that both these muscles make up only about 30% of tissue in duodenum and about 55% in ileum.

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† British Council Fellow.

Iron supplies in foetal and newborn rats.

By W. F. WIDDAS. *Physiology Department, St Mary's Hospital Medical School, Paddington*

It is generally thought that the full-term foetus is provided with a store of iron to tide it through the nursing period. Experiments carried out on rats using tracer technique with Fe^{59} indicate that there is no evidence of the storage of iron in the full-term foetus as a special mechanism, and that a maternal rat may supply twice as much iron to her young via the milk in the first 21 days following birth as she supplies during the whole of pregnancy.

The rate of supply is estimated at 25 mg./kg. weight increase. The iron/weight ratio at birth is about twice this value, and the ratio progressively falls to a minimum at 21–28 days post-partum. Possible factors are (i) rapid weight increase and magnitude of iron commitment, and (ii) progressive reduction in the proportion of vascular iron.

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